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Method for the production of evolved microorganisms which permits the generation or modification of metabolic pathways

5 This invention concerns a new method for the preparation of evolved microorganisms permitting the creation or modification of metabolic pathways, the strains of evolved microorganisms thereby obtained, the evolved genes coding for the evolved proteins that may be obtained by the method according to the invention, and the use of said evolved microorganisms, genes or proteins in a biotransformation
10 process.

 The preparation of microorganisms with modified properties is a widely used process. The aim is either to cause the microorganisms to evolve by letting them grow on a growth medium with a factor that exerts a selection pressure, so as to select those microorganisms able to resist that pressure, or to introduce one or more
15 heterologous genes by means of widely used genetic engineering methods, in order to lend the microorganisms new phenotypic features associated with the expression of said heterologous gene or genes. This evolution can be favored by the use of mutagenic agents well known to those skilled in the art.

 Methods for evolution by growth under selection pressure by removing a
20 gene necessary for the transformation of a component of the culture medium, and by means of a mutagenic agent, are described in particular in FR 2 823 219 and WO 03/004656.

Brief description of the invention

 This invention concerns a new method for the preparation of evolved
25 microorganisms permitting the creation or modification of metabolic pathways, characterized in that it comprises the following steps:

- a) Modification of the cells of an initial microorganism so as to inhibit the production or consumption of a metabolite when that microorganism is grown on a defined medium, thereby adversely affecting the growth
30 capacity of the microorganism. If the cells are not modified, the microorganism is able to produce or consume this metabolite, and displays a normal growth when it is grown on that same defined medium.

- b) Growth of the modified microorganism previously obtained on the said defined medium that caused it to evolve, where that defined medium can contain a co-substrate necessary for that evolution.
- c) Selection of the modified microorganism able to grow on the defined medium, with a co-substrate if appropriate.

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The evolved microorganism preferentially contains at least one evolved gene coding for an evolved protein, the evolution of which makes it possible to replace the inhibited metabolic pathway by a new metabolic pathway.

This invention also concerns a method comprising an additional step a1) in which at least one heterologous gene coding for a heterologous protein is introduced, which heterologous gene is intended to cause the evolution of a new metabolic pathway, prior to step b) in which a modified microorganism is grown.

This invention also concerns a method comprising a step d) in which an evolved gene coding for said evolved protein is isolated.

This invention also concerns a method according to the invention whereby the evolved gene obtained previously is introduced, in an appropriate form, into a production microorganism intended for the production of the evolved protein.

This invention also concerns an evolved microorganism that may be obtained by a method according to the invention as defined above and below.

The invention also concerns a method for the preparation of an evolved protein characterized in that an evolved microorganism according to the invention is grown in an appropriate culture medium for the production of the evolved protein, which protein is purified when appropriate.

This invention also concerns an evolved gene coding for an evolved protein that may be obtained by a method according to the invention as defined above and below.

This invention also concerns an evolved protein that may be obtained by a method according to the invention as defined above and below.

This invention also concerns the use of an evolved microorganism or an evolved protein as defined above and below in a biotransformation process.

Definitions

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According to the invention an 'evolved microorganism' is defined as a microorganism obtained by selection of a modified microorganism. The evolved microorganism displays at least one difference from the modified microorganism. This difference may, for example, be the improvement of an enzymatic characteristic, or the creation of a new metabolic pathway.

According to the invention a 'metabolic pathway' is one or more enzymatic reactions the succession of which forms a molecule (product) that is different from the starting molecule (substrate).

According to the invention a 'modification' is a change, in particular a deletion, of at least one gene and/or its promoter sequence, which gene codes for an enzyme.

According to the invention a 'metabolite' is a molecule synthesized and/or transformed by the microorganism.

According to the invention a 'defined medium' is a medium of known molecular composition suitable for the growth of the microorganism. The defined medium is substantially free of the metabolite or metabolites, the production of which is inhibited by performing the modification.

According to the invention a 'co-substrate' is an organic or inorganic molecule, different from the substrate, which is involved in a reaction and gives one or more of its atoms to the substrate to form a product. The co-substrate has no recognized mutagenic properties.

According to the invention 'selection' is a culture method used to select microorganisms that have evolved in such a way that a modification does not affect growth anymore. A preferred application is a continuous culture method, carried out by applying increasing rates of dilution so as to conserve in the culture medium only those microorganisms with a growth rate equal to or greater than the imposed rate of dilution.

According to the invention an 'evolved gene' is a sequence of nucleic acids (comprising A, T, G or C) bounded by a stop codon (TAA, TAG, TGA) in phase and possessing, after selection, at least one nucleic acid that is different from the initial sequence, so that the protein coded by that evolved gene differs in at least one amino acid from the protein coded by the initial gene.

According to the invention a 'heterologous gene' is a sequence of nucleic acids bounded by a start codon (ATG or GTG) and a stop codon (TAA, TAG, TGA) in phase, called a coding sequence, derived from an organism different from that used to carry out the evolution and/or the production.

5 According to the invention an 'evolved protein' is a sequence of amino acids (protein sequence) that differs in at least one amino acid from the initial protein sequence after selection.

According to the invention a 'heterologous protein' is a protein resulting from the translation of a heterologous gene.

10 The genes and proteins can be identified by their primary sequences, but also by sequence or alignment homology defining groups of proteins.

PFAM (protein families database of alignments and hidden Markov models; <http://www.sanger.ac.uk/Software/Pfam/>) represents a large collection of protein sequence alignments. Each PFAM makes it possible to visualize multiple alignments, 15 see protein domains, evaluate distribution among organisms, gain access to other databases, and visualize known protein structures.

COGs (clusters of orthologous groups of proteins; <http://www.ncbi.nlm.nih.gov/COG/>) are obtained by comparing protein sequences from 43 fully sequenced genomes representing 30 major phylogenic lines. Each 20 COG is defined from at least three lines, which permits the identification of former conserved domains.

The means of identifying homologous sequences and their percentage homologies are well known to those skilled in the art, and include in particular the BLAST programs, which can be used from the website 25 <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters indicated on that website. The sequences obtained can then be exploited (e.g., aligned) using, for example, the programs CLUSTALW (<http://www.ebi.ac.uk/clustalw/>) or MULTALIN (<http://prodes.toulouse.inra.fr/multalin/cgi-bin/multalin.pl>), with the default parameters indicated on those websites.

30 Using the references given on GenBank for known genes, those skilled in the art are able to determine the equivalent genes in other organisms, bacterial strains, yeasts, fungi, mammals, plants, etc. This routine work is advantageously done using

consensus sequences that can be determined by carrying out sequence alignments with genes derived from other microorganisms, and designing degenerate probes to clone the corresponding gene in another organism. These routine methods of molecular biology are well known to those skilled in the art, and are described, for example, in Sambrook *et al.* (1989 Molecular Cloning: a Laboratory Manual. 2nd ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.).

The genes that may be deleted or overexpressed for the evolved strains according to the invention are principally defined using the denomination of the gene of *E. coli*. However, this usage has a more general meaning according to the invention and covers the corresponding genes of other microorganisms. Using the GenBank references for the genes of *E. coli* those skilled in the art can determine the equivalent genes in bacterial strains other than *E. coli*.

According to the invention a 'new metabolic pathway' is a set of one or more enzyme reactions, the succession of which produces a chemical entity that, after the step to select the evolved microorganism, differs in its enzymatic activities from the corresponding pathway in the corresponding non-evolved microorganism. This difference can reside in the type of reaction catalyzed, or in kinetic characteristics (K_m , V_{max} , K_i , etc.). A new enzymatic pathway makes it possible to produce a chemical entity different from or the same as the initial product, from a substrate different from or the same as the initial substrate.

According to the invention an 'appropriate form' is a sequence of nucleic acids, bounded by a start codon (ATG or GTG) and a stop codon (TAA, TAG, TGA) in phase, called a coding sequence, or a part of that coding sequence, under the control of regulators necessary for its expression in the microorganism in which the heterologous gene is to be expressed. These regulators are well known to those skilled in the art, and include promoting regulators, or promoters, in particular promoters called strong constitutive promoters in microorganisms. The constitutive promoter is preferably chosen from among pTAC-O, pLAC-O, pTRC-O, strong promoters for which the lac operator has been deleted to make them constitutive, pTHLA.

According to the invention an 'initial microorganism' is a microorganism that has not yet undergone any modification, mutation or evolution.

According to the invention a 'production microorganism' is an evolved microorganism or optimized microorganism into which a new metabolic pathway from an evolved microorganism has been introduced.

According to the invention a 'modified microorganism' is a microorganism
5 obtained by performing controlled modifications, i.e., that are not the result of a process of evolution. Examples of such a modification are the directed mutation or deletion of a gene, or the directed modification of a promoter.

According to the invention a 'culture medium suitable for the production of the evolved protein' is a medium of defined composition, or a complex medium, or a
10 partially defined medium. The complex medium is obtained from a plant, microorganism or animal hydrolysate; its composition may be determined by analysis, although an exhaustive analysis of this type of medium is seldom possible. A partially defined medium is a defined medium to which a complex medium has been added.

15 According to the invention a 'biotransformation process' is a process whereby a molecule A is transformed into a molecule B by means of one or more enzymes, which may or may not be contained in one or more microorganisms. There are three types of biotransformation: bioconversion, fermentation and biocatalysis. In bioconversion, the enzyme or enzymes are produced in one or more microorganisms
20 grown on a suitable medium, and substance A and if necessary one or more co-substrates are supplied for conversion into substance B. In fermentation, the enzyme or enzymes are produced in one or more microorganisms grown on a suitable medium to enable the microorganism or microorganisms to synthesize substance A; the suitable medium can contain co-substrates. In biocatalysis, the enzyme or
25 enzymes are not in cells but in a suitable medium supplying substance A and any co-substrates necessary for the biotransformation.

The methods for the isolation of genes are well known to those skilled in the art, and are described in particular in Sambrook *et al.* (1989 Molecular Cloning: a Laboratory Manual. 2nd Ed. Cold Spring Harbor Lab., Cold Spring Harbor, New
30 York), Ausubel *et al.*, 1987 (Current Protocols in Molecular Biology, John Wiley and Sons, New York); Maniatis *et al.*, 1982, (Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). These

methods make it possible to locate, copy or extract the gene in order to introduce it into a new organism. This last step can be preceded by a step in which the gene is incorporated into a polynucleotide before being introduced into the microorganism.

5 Methods for purifying proteins are well known to those skilled in the art, and are described in particular in Coligan *et al.*, 1997 (Current Protocols in Protein Science, John Wiley & Sons, Inc). They make it possible to identify a protein of interest in a fractionated or non-fractionated protein extract. This protein can then be purified, resulting in an increase in its specific activity if it is an enzyme. Lastly, these methods make it possible, if necessary, to immobilize the protein on a support
10 (e.g., a resin).

According to the invention a 'deletion' is the suppression of the activity of the 'deleted' gene. This suppression can result from an inactivation, by suitable means, of the product of the expression of the gene concerned, or the inhibition of the expression of the gene concerned, or the deletion of at least part of the gene
15 concerned so that its expression is impaired (for example deletion of part or all of promoter region necessary for its expression), or the loss of function of the product of the expression (for example deletion in the coding part of the gene concerned). The deletion of a gene preferably consists of the removal of most of that gene, and if appropriate its replacement by a selection marker gene to facilitate identification,
20 isolation and purification of the evolved strains according to the invention.

According to the invention a 'substrate' is a metabolite that can be transformed by the action of an enzyme, if necessary in the presence of a co-substrate.

Detailed description of the invention

A. Modified microorganisms

25 The strains of modified microorganisms according to the invention can be prokaryotic or eukaryotic.

According to the invention a strain of a microorganism is a set of microorganisms belonging to the same species, that comprises at least one
30 microorganism of that species. Thus the characteristics described for the strain apply to each of the microorganisms of that strain. Likewise, the characteristics described

for one of the microorganisms of the strain apply to all the microorganisms of which that strain is composed.

The optimized bacteria according to the invention are selected from among bacteria, yeasts and fungi, in particular among the following species: *Aspergillus sp.*,
 5 *Bacillus sp.*, *Brevibacterium sp.*, *Clostridium sp.*, *Corynebacterium sp.*, *Escherichia sp.*, *Gluconobacter sp.*, *Pseudomonas sp.*, *Rhodococcus sp.*, *Saccharomyces sp.*, *Streptomyces sp.*, *Xanthomonas sp.*, *Candida sp.*

In a preferred embodiment the bacterial strain is a strain of *Escherichia*, in particular *E. coli*. In another embodiment the bacterial strain is a strain of
 10 *Corynebacterium*, in particular *C. glutamicum*.

In another embodiment the yeast strain is a strain of *Saccharomyces*, in particular *S. cerevisiae*

To prepare such modified microorganisms it can be advantageous to attenuate, and in particular to delete, other genes associated with or independent of
 15 the metabolic pathway to be modified, in order to favor the evolution of the microorganism.

To prepare such modified microorganisms it can also be advantageous to favor, and in particular to overexpress, other heterologous or non-heterologous genes, associated with or independent of the metabolic pathway to be modified, in
 20 order to favor the evolution of the microorganism.

The overexpression of a gene can be achieved by replacing the promoter of that gene *in situ* by a strong or inducible promoter. Alternatively, a single-copy or multicopy replicative plasmid in which the gene that is to be overexpressed is controlled by the appropriate promoter is introduced into the cell.

25 Such modifications will be decided on case by case according to the choice of metabolic pathway to be modified. In particular they will be described case by case for the particular metabolic pathways outlined below.

Those skilled in the art know the protocols used to modify the genetic characters of microorganisms.

30 The inactivation of a gene is carried out preferably by homologous recombination. (Datsenko, K.A.; Wanner, B.L. (2000), One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad.*

Sci. USA 97: 6640-6645). The principal of a protocol is briefly as follows: a linear fragment obtained *in vitro* is introduced into the cell; this fragment comprises the two regions flanking the gene and at least one gene of selection between these two regions (generally a gene of resistance to an antibiotic); the fragment therefore presents an inactivated gene. The cells that have undergone a recombination event and have integrated the fragment are then selected by spreading them on a selective medium. The cells that have undergone a double recombination event in which the native gene has been replaced by the inactivated gene are then selected. This protocol can be improved by using positive or negative selection systems to increase the rate of detection of double recombination events.

The inactivation of a gene in *S. cerevisiae* is achieved preferably by homologous recombination (Baudin *et al.*, *Nucl. Acids Res.* 21, 3329-3330, 1993; Wach *et al.*, *Yeast* 10, 1793-1808, 1994; Brachmann *et al.*, *Yeast* 14:115-32, 1998).

B. Production microorganisms

The production microorganisms are also selected from among the bacteria, yeasts and fungi listed above. They can be evolved microorganisms obtained by the evolution procedure according to the invention, or microorganisms optimized for the production of a desired metabolite, in which at least one evolved gene according to the invention has been introduced.

C. Culture of microorganisms

According to the invention, the terms 'culture' and 'fermentation' are used indifferently to denote the growth of a microorganism on an appropriate culture medium containing a simple carbon source.

According to the invention a simple carbon source is a source of carbon that can be used by those skilled in the art to obtain normal growth of a microorganism, in particular of a bacterium. In particular it can be an assimilatable sugar such as glucose, galactose, sucrose, lactose or molasses, or by-products of these sugars. An especially preferred simple carbon source is glucose. Another preferred simple carbon source is sucrose.

Those skilled in the art are able to define the culture conditions for the microorganisms according to the invention. In particular the bacteria are fermented at

a temperature between 20°C and 55°C, preferably between 25°C and 40°C, and more specifically about 30°C for *C. glutamicum* and about 37°C for *E. coli*.

The fermentation is generally conducted in fermenters with an inorganic culture medium of known defined composition adapted to the bacteria used,
5 containing at least one simple carbon source, and if necessary a co-substrate necessary for the production of the metabolite.

In particular, the inorganic culture medium for *E. coli* can thus be of identical or similar composition to an M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA* 32:120-128), an M63 medium (Miller, 1992; A Short Course in Bacterial
10 Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) or a medium such as that defined by Schaefer *et al.* (1999, *Anal. Biochem.* 270: 88-96).

Analogously, the inorganic culture medium for *C. glutamicum* can thus be of identical or similar composition to BMCG medium (Liebl *et al.*, 1989, *Appl.*
15 *Microbiol. Biotechnol.* 32: 205-210) or to a medium such as that described by Riedel *et al.* (2001, *J. Mol. Microbiol. Biotechnol.* 3: 573-583).

D. Metabolic pathways

The metabolic pathways to be evolved are generally selected from among the synthetic pathways of amino acids, the synthetic pathways of nucleic acids, the
20 synthetic pathways of lipids or the metabolic pathways of sugars.

In a first preferred embodiment of the invention, the evolved metabolic pathway is a biosynthesis pathway of amino acids, in particular a biosynthesis pathway of an amino acid selected from among methionine, cysteine, threonine, lysine, or isoleucine.

25 In a second preferred embodiment of the invention the modified metabolic pathway is a pathway by which NADP is regenerated from NADPH. In particular the biosynthesis pathway of cysteine, of hydroxypropionate and of xylitol will be cited.

D.I. The methionine biosynthesis pathway

The invention can be applied, for example, to the methionine biosynthesis
30 pathway (Figure 1) and yields strains of microorganisms, in particular bacteria, e.g., *E. coli* and corynebacteria, that produce 2-amino-4-(alkylmercapto)butyric acid, in particular L-methionine 2-amino-4-(methylmercapto)butyric acid) by metabolism of a

simple carbon source and a sulfur source, in particular methylmercaptan (CH_3SH), hydrogen sulfide (H_2S) or physiologically acceptable salts of these. The sulfur source H_2S can also be introduced into the culture medium as sulfate. The invention also concerns the strain of a microorganism, the improved enzymes and their coding sequences. The invention concerns finally a process for the preparation of methionine by culturing the said strain of a microorganism. DL-methionine is produced industrially by chelical synthesis. Methylmercaptan reacts with acroleine to produce β -thiopropionaldehyde that reacts with hydrogen cyanide producing α -hydroxy- γ -thiobutyronitrile. After treatment with ammonia and hydrolysis methionine is obtained.

All the industrial producers of DL-methionine use the same raw materials, namely acrolein, methane thiol (methyl mercaptan), hydrogen cyanide and ammonia or ammonium carbonate. This synthesis of racemic methionine can be carried out as a batch or a continuous process.

One industrial process includes biocatalysis in the chemical synthesis, using amino acylase, an enzyme produced by *Aspergillus oryzae*, to obtain pure L-methionine from DL-methionine

Patents US 6,379,934 and EP 1 055 730 describe the production of amino acids using a strain of coryneform bacteria in which the *accBC* gene is amplified. Methionine is mentioned, but only the preparation of L-lysine is exemplified.

However, the synthesis of sulfur-containing amino acids by culture of microorganisms remains difficult to implement at a scale suited to possible industrial production, in particular owing to the complexity of their biosynthesis pathways and their many mechanisms of regulation.

The metabolism of methionine is closely regulated at several levels (Weissbach et al., 1991, Mol. Microbiol., 5, 1593-1597):

- Carbon metabolism for the synthesis of L-serine from 3-Phosphoglycerate, and L-homoserine from aspartate and acetyl-CoA.
- Sulfur metabolism for the synthesis of L-cysteine from L-serine, acetyl-CoA and sulfate present in the culture medium.

- Methionine synthesis (Fig. 1) from L-homoserine, cysteine and acetyl-CoA or succinyl-CoA.

Patent application WO 93/17112 describes the different enzymes involved in the biosynthesis of methionine from L-aspartic acid in various organisms. This patent
 5 application also describes the introduction into a micro-organism of several exogenous genes acting in sequence for the synthesis of methionine, using methyl mercaptan or hydrogen sulfide as a sulfur source.

This invention concerns strains of micro-organisms, in particular of bacteria, in particular of *E. coli* and corynebacteria, producing 2-amino-4-
 10 (alkylmercapto)butyric acids of general formula (I)



where R is a straight-chain or branched-chain alkyl group containing 1 to 18 carbon atoms, which may bear one or more hydroxy substituents, or an aryl group or a heteroaryl group containing one or more atoms of nitrogen or sulfur in its
 15 heteroaromatic ring, and which can be a phenyl, pyridyl, pyrrolyl, pyrazolyl, triazolyl, tetrazolyl, thiazolyl, or thienyl group,

by the metabolism of a simple carbon source and a sulfur source consisting of a compound of general formula (II)



20 where R' is a hydrogen atom or a group R as defined above, and its physiologically acceptable salts,

which strains present at least one gene coding for an enzyme with a modified 'methionine synthase' activity.

An enzyme with a modified 'methionine synthase' activity according to the
 25 invention is any enzyme involved in the biosynthesis of 2-amino-4-(alkylmercapto)butyric acids of general formula (I) by bioconversion of a substrate of general formula (III)



where R'' is an acyl group, preferably either a succinyl group or an acetyl
 30 group,

or by direct bioconversion of the substrate into an acid of general formula (I), or by bioconversion of the substrate into homocysteine, of formula (IV)



which is then converted into the acid of general formula (I) by an appropriate enzyme.

The enzymes with modified 'methionine synthase' activity are enzymes that are modified compared with the wild type enzymes, so that they will preferentially perform the direct bioconversion of the substrate of general formula (III) into the acid of general formula (I) or homocysteine, of formula (IV), in place of the reaction catalysed by the wild type enzyme. This modification, also termed mutation, results essentially in a greater affinity of the modified enzyme for the sulfur-containing compound of general formula (II) than for its natural co-substrate.

A simple carbon source according to the present invention is any carbon source that can be used by those skilled in the art to obtain the normal growth of a micro-organism, in particular a bacterium. This definition includes in particular any assimilable sugar, such as glucose, galactose, sucrose, or molasses, or by-products of these sugars. One especially preferred simple carbon source is glucose. Another preferred simple carbon source is sucrose.

A physiologically acceptable salt according to the invention is any salt of the compound of general formula (I) that does not affect the metabolism or the growth capacity of the micro-organism according to the invention, being in particular an alkali metal salt, such as a sodium salt.

In a preferred embodiment of the invention, R is a straight-chain or branched-chain alkyl group containing 1 to 4 atoms of carbon, in particular a methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl or t-butyl group, and is preferably a methyl group.

The 2-amino-4-(alkylmercapto)butyric acid of general formula (I) obtained is preferably L-methionine. The use of a simple carbon source and a sulfur compound of formula (II) to produce an acid of formula (I) by bioconversion offers several advantages *a priori*, in particular:

- The synthesis of the acid (I), and of methionine, in one or two steps from O-acyl-L-homoserine, becomes independent of the synthesis of cysteine, and also of the tetrahydrofolate cycle.
- The sulfur-containing compounds of formula (II), such as methyl mercaptan, which are generally toxic petrochemicals, are used to good

purpose as raw materials for the biosynthesis of useful amino acids with added value.

The invention is based on the directed modification of the 'methionine-synthase' activity of cystathionine- γ -synthase (EC 4.2.99.9; GenBank AAN83320, or AAA24167) in the presence of methyl-mercaptan. This enzyme of the methionine biosynthesis pathway, coded for by the gene *metB* in *E. coli* (Fig. 2) and *C. glutamicum*, presents an activity for a wide spectrum of substrates (Flavin, M.; Slaughter, C. (1967) Enzymatic synthesis of homoserine or methionine directly from O-succinyl-homoserine. Biochim. Biophys. Acta 132: 400-405).

The invention is also based on the directed modification of the 'methionine-synthase' activity of O-acetyl-L-homoserine sulphydrylase (or O-acetyl-L-homoserine sulphydrylase, C 4.2.99.10) in the presence of methyl mercaptan. This enzyme of the methionine biosynthesis pathway, coded for by the gene *metY* in *C. glutamicum* (Genbank AF220150), presents an activity for a wide spectrum of substrates (Smith IK, Thompson JF. (1969) Utilization of S-methylcysteine and methyl mercaptan by methionineless mutants of Neurospora and the pathway of their conversion to methionine. II. Enzyme studies. Biochim Biophys Acta 184(1):130-8).

The invention therefore also concerns a method for the preparation of the strains according to the invention and their use in a method for the preparation of a 2-amino-4-(alkylmercapto)butyric acid of general formula (I), preferably L-methionine.

The method for the preparation of strains according to the invention consists of obtaining, from an initial bacterial strain, a genetically modified bacterial strain that presents at least one modification to a gene coding for an enzyme with 'methionine synthase' activity, by a process that comprises a step in which that initial bacterial strain is subjected to a selection pressure in the presence of a compound of formula (II) defined above, in order to direct the evolution of the gene coding for the enzyme with 'methionine synthase' activity in that bacterial strain towards a gene coding for an enzyme with a 'methionine synthase' activity that is modified relative to that of the initial bacterial strain.

A 'methionine synthase' activity is improved in strain (A) of the micro-organism relative to the initial strain (I) when the production of methionine in the same culture conditions (in a medium containing an appropriate quantity of the sulfur-containing compound of formula (II)) is greater in strain (A) than in strain (I).

5 This improvement is preferably observed by measuring the quantity of methionine produced. In some cases this improvement can be observed by the increase in the growth rate of bacteria (A) relative to the growth rate of bacteria (I), in a minimum medium containing no methionine.

This invention also concerns those strains with improved 'methionine
10 synthase' activity that can be obtained by the selection process according to the invention, and comprising at least one gene coding for an enzyme with a modified 'methionine synthase' activity as defined above and below.

In a first embodiment of the invention the enzyme with modified 'methionine
15 synthase' activity allows the direct conversion of the substrate of general formula (III) into the acid of general formula (I). In this case the sulfur source is a compound of general formula (II) where R' is a group R as defined above.

The enzyme with modified methionine synthase activity is chosen from among the cystathionine- γ -synthases and the acylhomoserine sulphydrylases.

20 In a second embodiment of the invention the enzyme with modified 'methionine synthase' activity allows the conversion of the substrate of general formula (III) into homocysteine, of general formula (IV)



which is then converted into an acid of general formula (I) by an appropriate
25 enzyme. In this case, the sulfur-containing source is a compound of general formula (II) in which R' is an atom of hydrogen, preferably, hydrogen sulfide. The sulfur source H_2S can be introduced into the culture medium or be produced by the bacteria from a simple sulfur source, for example a sulfate.

The enzyme with modified methionine synthase activity is advantageously
30 chosen from among the cystathionine- γ -synthases and the acylhomoserine sulphydrylases.

Those skilled in the art will know how to select other enzymes with modified 'methionine synthase' activity according to their ability to evolve, so as to carry out the modified enzymatic 'methionine synthase reaction' as defined above. Examples include in particular the cysteine synthases A and B, coded for by the genes *cysK* and *cysM* in bacteria.

For the modified cystathionine- γ -synthases defined above and below, the substrate is advantageously O-acetyl-L-homoserine or O-succinyl-L-homoserine, preferably O-succinyl-L-homoserine.

For the modified acylhomoserine sulfhydrylases defined above and below, the substrate is advantageously O-succinyl-L-homoserine or O-acetyl-L-homoserine, preferably O-acetyl-L-homoserine.

For these two enzymes, the modification consists essentially of a mutation such that the conversion of the substrate of general formula (III) takes place preferentially with the compound of general formula (II) rather than with L-cysteine.

The mutation of the enzymes can be obtained by implementing the method for the preparation of strains with improved 'methionine synthase' activity according to the invention by culture under selection pressure in the presence of the compound of general formula (II).

In this case, the gene with the sequence coding for cystathionine- γ -synthase or acylhomoserine sulfhydrylase can be

- a wild type gene present in the genome of the original strain (I), where it is expressed to allow the translation of the corresponding enzyme, or
- a heterologous gene that includes a sequence coding for a cystathionine- γ -synthase or an acylhomoserine sulfhydrylase, under the control of regulatory elements that permit its expression and translation in the original strain (I) into which it was introduced, or
- obtained by directed mutagenesis directly on the wild type gene present naturally in the initial strain (I), in particular by homologous recombination, or
- obtained by the usual techniques of directed mutagenesis on sequences coding for a cystathionine- γ -synthase or an acylhomoserine sulfhydrylase, subsequently introduced into the initial strain (I) under the control of regulatory elements that

allow its expression and translation in the initial strain (I) into which it was introduced.

Such regulatory elements are well known to those skilled in the art, and contain promoter regulation sequences, or promoters, in particular strong constitutive promoters in micro-organisms. The strong constitutive promoter is preferably
 5 pTAC-O (SEQ ID No. 1), or pLAC-O (SEQ ID No. 2), or pTRC-O (SEQ ID No. 3), or pTHLA (SEQ ID No. 4), all of which are strong promoters in which the lac operator has been deleted to make them constitutive.

The 'initial' or unmodified 'methionine synthase' activity cystathionine- γ -
 10 synthases are advantageously selected among the cystathionine- γ -synthases corresponding to PFAM reference PF01053 and to COG reference CPG0626.

The cystathionine- γ -synthase is preferably the cystathionine- γ -synthase from *E. coli* K12, represented by SEQ ID No. 6. Homologous sequences of this sequence have cystathionine- γ -synthase activity and at least 80% homology, preferably 90%
 15 homology, and more preferably 95% homology with the amino acid sequence of SEQ ID No. 6.

The means of identifying homologous sequences and their percentage of homology are well-known to those skilled in the art, and include in particular the BLAST program, and in particular the BLASTP program, which can be used from
 20 the web site <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters indicated on that site.

In a preferred embodiment of the invention the initial cystathionine- γ -synthase, before modification, contains the following sequence of amino acids in its C-terminal part. (conserved zone 1)

25 X1-X2-X3-L-G-X4-X5-X6-X7-X8-X9

in which

X1 represents A,G,S, preferably A

X2 represents E,V,P,T, preferably E

X3 represents S,T,N, preferably S

30 X4 represents G,D,A,H,T, preferably G

X5 represents V,A,T,H,N, preferably V

X6 represents E,R,K,F, preferably E

X7 represents S,T, preferably S

X8 represents L,I,V,A, preferably L and

X9 represents I,V,A,T, preferably I.

- 5 The cystathionine- γ -synthase with modified 'methionine synthase' activity according to the invention preferably contains the following amino acid sequence in its C-terminal part:

A-E-S-L-G-G-V-E-S

- 10 The initial cystathionine γ -synthase, before modification, also advantageously contains at least one of the following amino acid sequences in its N-terminal part (conserved zone 2):

X10-X11-Y-X12-R-X13-X14-X15-X16-X17-X18

in which

- 15 X10 represents H,Y,F,L,K, preferably A
 X11 represents E,D,K,R,V,I, preferably Y
 X12 represents S,A,T,P,G, preferably S
 X13 represents I,S,T,R,E,F,W,D, preferably S
 X14 represents S,G,A,I,E,N,K,P, preferably G
 X15 represents N,H,Q,S, preferably N
 20 X16 represents P,D,L, preferably P
 X17 represents T,M,N,G,S, preferably T and
 X18 represents R,L,V,S,W,E, preferably R.

- 25 Amino acids X1 to X9 correspond to residues 324 to 334 of the cystathionine γ -synthase sequence of *E. coli* K12, represented on SEQ ID No. 6.

- The amino acids X10 to X18 correspond to residues 44 to 54 of the sequence of cystathionine γ -synthase of *E. coli* K12, represented on SEQ ID No. 6.

- The positions of the amino acids used above and below are relative positions set with reference to the sequence of the cystathionine- γ -synthase of *E. coli* K12.
 30 Those skilled in the art will of course know how to find the corresponding amino acids on other sequences of cystathionine- γ -synthases, using the usual sequence

alignment tools. These tools include in particular the BLAST program, which can be used from the web site <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters indicated on that site. The sequence alignment can be carried out at the level of both the protein (SEQ ID No. 6) and its coding sequence, such as for example the coding sequence represented on SEQ ID No. 5.

The advanced search function of BlastP can also be advantageously used by refining the search with a (PHI-BLAST) motif. In this case the motif [AGS]-[EVPT]-[STN]-**L-G**-[GDAHT]-[VATHN]-[ERKF]-[ST]-[LIVA]-[IVAT] can be taken for a first conserved zone, and the motif x(2)-Y-[SATPG]-R-x(2)-[NHQS]-[PDL]-[TMNGS]-[RLVSWE] for a second conserved zone, where the letters in bold type represent the amino acids found most often at that position in the sequence, and where x stands for any amino acid. The number 2 in brackets signifies that there are two undetermined amino acids.

For the sequence alignment, the CLUSTALW program (<http://www.ebi.ac.uk/clustalw/>) or the MULTALIN program (<http://prodes.toulouse.inra.fr/multalin/cgi-bin/multalin.pl>) can be used, with the default parameters indicated on those sites.

One such sequence alignment is represented in Figure 7 for a selection of different cystathionine- γ -synthases.

These are preferably chosen from among the following cystathionine- γ -synthases (CGS):

Q9ZMW7 O-succinyl homoserine (thiol)-lyase, *Helicobacter pylori*

P46807 O-succinyl homoserine (thiol)-lyase, *Mycobacterium leprae*

AAO29646 *Xylella fastidiosa* Temecula1

25 NP_638204 *Xanthomonas campestris* pv. *campestris* str. ATCC 33913

NP_358970 *Streptococcus pneumoniae* R6

NP_126586 O-succinylhomoserine (thiol)-lyase, *Pyrococcus abyssi*

NP_373671 *Staphylococcus aureus* subsp. *aureus* N315

NP_418374 [*Escherichia coli* K12

30 NP_601979 *Corynebacterium glutamicum* ATCC 13032

NP_343729 O-succinylhomoserine (thiol)-lyase, *Sulfolobus solfataricus*

NP_786043 O-succinylhomoserine (thiol)-lyase, *Lactobacillus plantarum* WCFS1

- NP_719586 *Shewanella oneidensis* MR-1
 CAD30944 *Streptomyces coelicolor* A3(2)
 NP_696324 *Bifidobacterium longum* NCC2705
 NP_457953 *Salmonella enterica* subsp. *enterica* serovar *typhi*
 5 NP_539021 *Brucella melitensis*
 EAA30199 O-succinylhomoserine (thiol)-lyase, *Neurospora crassa*
 BAC61028 *Vibrio parahaemolyticus*.

The modified cystathionine- γ -synthase as defined above preferably presents
 10 at least one mutation in its C-terminal part, and (or) at least one mutation in its N-terminal part.

A mutation according to the invention is the substitution of an amino acid in the wild type sequence by a different amino acid.

The mutation preferably consists of the replacement of an acidic amino acid,
 15 which interacts with the cysteine co-substrate for the unmodified enzyme, by an apolar amino acid. This apolar amino acid is a glycine, alanine, leucine, isoleucine, valine, phenylalanine or methionine residue.

These amino acids can be identified by reference to the crystal structure of the cystathionine- γ -synthases of *E. coli*, described by Clausen et al. (EMBOJ, Vol.
 20 17, No. 23, pp 6827-6838, 1998).

The mutation in the C-terminal part is advantageously introduced among the acidic amino acids of the 'conserved zone 1' as defined above, in particular at the level of residue X2.

The cystathionine- γ -synthase with modified 'methionine synthase' according
 25 to the invention advantageously contains the following amino acid sequence in its C-terminal part:

X1-X2-X3-L-G-X4-X5-X6-X7-X8-X9

in which

X1, X3, X4, X5, X6, X7, X8 and X9 are defined above, and
 30 X2 stands for G,A,L,I,V,F, or M, and preferably A.

The mutation in the N-terminal part is advantageously introduced among the amino acids of the 'conserved zone 2' as defined above, in particular at the level of residue X11 and (or) R and (or) X13.

Preferentially the cystathionine- γ -synthase with modified 'methionine synthase' activity according to the invention contains the following amino acid sequence in its C-terminal part.

A-A-S-L-G-G-V-E-S

The cystathionine- γ -synthase with modified 'methionine synthase' activity according to the invention can advantageously contain the following amino acid sequence in its N-terminal part:

X10-X11-Y-X12-X19-X13-X14-X15-X16-X17-X18

in which

X7, X9, X12, X14, X15, X16, X17 and X18 are defined above,

X11 is defined above or is an apolar amino acid,

15 X13 is defined above or is an apolar amino acid,

X19 is defined above or is an apolar amino acid, and

at least one of the amino acids X11, X13 and X19 is an apolar amino acid as described above.

In a preferred embodiment of the invention the cystathionine- γ -synthase with modified 'methionine synthase' activity according to the invention is a cystathionine- γ -synthase as defined above, modified to allow the direct conversion of O-succinyl-L-homoserine into L-methionine with methyl mercaptan as a sulfur source (compound of general formula (II) where R is a methyl group).

In a preferred embodiment of the invention the cystathionine- γ -synthase with modified 'methionine synthase' activity contains the sequence of amino acids represented on SEQ ID No. 8. A DNA sequence coding for this modified enzyme is represented on SEQ ID No. 7.

This invention also concerns the modified cystathionine- γ -synthases as defined above, and the nucleic acid sequences coding for these modified cystathionine- γ -synthases, in particular the isolated sequences, in particular the DNA sequences, and in particular the sequence represented on SEQ ID No. 7, the cloning

and (or) expression vectors containing those nucleic acid sequences under the control of the regulatory elements necessary for the expression and transcription of the modified cystathionine- γ -synthase in a host organism, and the host organisms transformed with those vectors.

5

The 'initial' or unmodified 'methionine synthase' activity acylhomoserine sulfhydrylases are advantageously chosen among the acylhomoserine sulfhydrylases corresponding to the PFAM reference PF01053 and the COG reference COG2873.

They are preferably chosen among the following acylhomoserine
10 sulfhydrylases:

NP_785969 O-acetylhomoserine (thiol)-lyase, *Lactobacillus plantarum* WCFS1

AAN68137 O-acetylhomoserine sulfhydrylase, *Pseudomonas putida* KT2440

NP_599886 O-acetylhomoserine sulfhydrylase, *Corynebacterium glutamicum*
ATCC 13032

15 NP_712243 acetylhomoserine sulfhydrylase, *Leptospira interrogans* serovar lai str.
56601

BAC46370 O-succinylhomoserine sulfhydrylase, *Bradyrhizobium japonicum*
USDA110

AAO57279 O-succinylhomoserine sulfhydrylase, *Pseudomonas syringae* pv.
20 tomato str. DC3000

NP_284520 O-succinylhomoserine sulfhydrylase [*Neisseria meningitidis* Z2491

AAA83435 O-succinylhomoserine sulfhydrylase (*P. aeruginosa*)

In a preferred embodiment of the invention, the modification of the methionine biosynthetic pathway requires initially a genetically modified bacterium.

25 The modification involves necessarily the attenuation of at least one gene and possibly the cloning of at least one heterologous gene. The attenuation of the gene makes the bacterium dependent on the restoration of an equivalent metabolic pathway allowing its growth. The genetically modified bacterium is cultivated and the strain or the bacterial strains are selected whose growth rate improves in the
30 presence or absence of an exogenous co-substrate.

The process for the construction of strains according to the invention consists of obtaining, starting with an initial strain, a genetically modified bacterial strain that

comprises at least one modification in a gene coding for an enzyme with “methionine synthase” activity (e.g. *E. coli* MetE) or “homocysteine synthase” activity (e.g. *E. coli* MetC). This process comprises a step that consists of subjecting the said initial bacterial strain to a selection pressure in the presence of the sulfur source defined
5 above in order to direct the evolution of at least one gene (e.g. *metB* in *E. coli*) in the said bacterial strain, in order to restore a methionine synthase activity or homocysteine synthase activity in the evolved strain; this restoration of activity is not linked to a reversion of the effected modification.

The invention also concerns a strain with improved “methionine synthase”
10 activity that comprises an inactivation of at least one endogenous gene involved in the conventional methionine biosynthesis pathway.

In a preferred embodiment the bacterial strain comprises an inactivation of at least one endogenous gene. This endogenous gene is chosen among *metB*, *metJ*, *metC*, *metE*, or *metH*.

15 In a preferred embodiment of the invention, the said modification of the initial strain that is found in the strain with improved “methionine synthase” activity, notably *E. coli* consists of inactivating the wild-type methionine synthase activity (encoded by the gene *metE* in *E. coli*) and then cultivating the said obtained modified microorganism on a defined medium lacking methionine, S-adenosylmethionine,
20 homocysteine and cystathionine, but containing methylmercaptan (exogenous co-substrate), or one of its salts, and of selecting an evolved microorganism with strongly improved methionine synthase activity of the cystathionine-γ-synthase, in the presence of methylmercaptan, to the point that it replaces the activity that was initially inactivated. In this embodiment of the invention it is important to note that
25 the medium used for the selection of the evolved microorganism is identical to the medium on which the initial microorganism (the one that is not modified) grows; only one co-substrate (e.g. alkylmercaptan) is added. In a specific embodiment of the invention, the bacterial strain selected comprising the evolved microorganism according to the invention, is an *E. coli* strain, more particularly the strain *E. coli*
30 K183, that has been registered under the number I-3005, on April, 2nd 2003 at the Collection Nationale de Culture et Microorganismes (CNCM), 25 rue du Docteur Roux, 75724 Paris Cedex 15, France according to the treaty of Budapest. This strain

comprises a gene expressing modified cystathionine- γ -synthase, the enzyme harboring the mutation E325A, and the inactivation of *metE*.

In a particular application of this invention, it is also possible to use strains that have additional deletions in the *metC* and/or *metH* and/or *metJ* to obtain the
5 strains with the corresponding improved "methionine synthase".

In a second equally preferred embodiment of the invention, the said modification of the initial strain, notably *E. coli*, consists of inactivating the cystathionine- β -lyase activity (encoded by the gene *metC* in *E. coli*), then of cultivating the said modified microorganism that is obtained on a defined medium
10 lacking methionine, S-adenosylmethionine, homocysteine and cystathionine, then selecting an evolved microorganism in which the homocysteine synthase activity of the cystathionine- γ -synthase is strongly improved in the presence of endogenous H₂S, to the point of replacing the initial inactivated activity. In this embodiment it is important to note that the medium used for the selection of the evolved
15 microorganism is identical to the medium on which grows the initial microorganism (the non-modified). In a specific embodiment of this application, it is also possible to add NaSH to the medium. In another specific application it is possible to use a strain that in addition has at least a mutation in *metH* and/or *metJ*.

In a third equally preferred embodiment of the invention, the said
20 modification of the initial strain, notably *E. coli*, consists of inactivating the wild type succinyl-homoserine sulfhydrylase (encoded by the *metB* gene) and methionine synthase (encoded by the *metE* gene in *E. coli*). Then the acetyl-homoserine sulfhydrylase (encoded by the *metY* gene in *C. glutamicum*) is introduced and the said modified obtained microorganism is cultured on a defined medium lacking
25 methionine, S-adenosylmethionine, homocysteine and cystathionine, but supplemented with sodium methylmercaptide with the goal to select an evolved microorganism in which the methionine synthase activity carried by the acetyl-homoserine sulfhydrylase (MetY) is strongly improved, in the presence of sodium methylmercaptide, to the point of replacing the methionine synthase activity that
30 was initially inactivated. In this embodiment of the invention it is important to note that the medium used for the selection of the evolved microorganism is identical to the medium on which the initial microorganism (the one that is not modified) grows;

only a co-substrate (e.g. methylmercaptan) is added. In a particular application of this invention, it is possible to use strains that have additional deletions in at least the *metH* and/or *metJ* and/or *metC* gene.

5 A mutation of the gene *metJ* is proposed in JP 2000157267-A/3, to produce a greater quantity of methionine (see also GenBank E35587). This gene codes for a protein that represses the genes *metB*, *E*, *L*, *J* and *R* (in *Salmonella typhimurium*). Its inactivation or its modification reduces the negative feedback control exerted by methionine.

10 The gene *metC* (GenBank M12858), codes for the cystathionine- β -lyase (EC 4.4.1.8), and the genes *metE* (GenBank AE000458) and *metH* (GenBank J04975) code for the methionine synthase (EC 2.1.1.13). Methionine is an amino acid that is essential for cell life. The inactivation of one or more of these genes has the effect of suppressing the usual methionine biosynthesis pathway.

15 Using the references given in GenBank for these genes, which are well known, those skilled in the art are able to determine the equivalent genes in bacterial strains other than *E. coli*. This routine work is advantageously performed using consensus sequences that can be determined from the synthesis of these genes for other micro-organisms, and by designing degenerate probes to clone the corresponding gene in another organism. These routine techniques in molecular
20 biology are well known to those skilled in the art, and are described for example in Sambrook et al. (1989 Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.).

The strain with modified 'methionine synthase' activity according to the invention that possesses an enzyme with modified 'methionine synthase' activity
25 defined above preferably presents at least one inactivation of the gene *metE* and (or) *metH*, and (or) of the gene *metC*, and (or) of the gene *metB*.

When the enzyme with modified 'methionine synthase' activity defined above allows the direct bioconversion of the substrate of general formula (III) into an acid of general formula (I), the strain according to the invention advantageously
30 presents at least one inactivation of the gene *metE* and (or) *metH* and (or) *metB*, preferably at least one inactivation of the gene *metE*.

When the enzyme with modified 'methionine synthase' activity defined above allows the direct bioconversion of the substrate of general formula (III) into homocysteine, of formula (IV)



5 which is then converted into an acid of general formula (I) by an appropriate enzyme, the strain according to the invention presents at least one inactivation of the gene *metC* and (or) *metB*. It can also present an inactivation of the gene *metE* and (or) endogenous *metH*. In this case, the methylase activity associated with the genes *metE* and (or) *metH* is restored by the introduction of a gene coding for an enzyme
10 with the same activity. This enzyme can be one that has been selected and (or) modified to improve the yields of the synthesis of the amino acids of general formula (I).

In one embodiment, the bacterial strain can also present a modification of the homoserine O-acyltransferase activity controlled by the gene *metA* such as to lend it
15 either a homoserine O-succinyltransferase activity (EC 2.3.1.46) or a homoserine O-acetyltransferase activity (EC 2.3.1.11).

In a particular embodiment, it is possible to replace or modify the gene *metA* of *E. coli*, coding for the enzyme possessing the homoserine O-succinyltransferase activity (Genbank AAN83396), so as to obtain a homoserine O-acetyltransferase
20 activity. Those skilled in the art know that this activity is coded for by the gene *metA* of *C. glutamicum* (Genbank AF052652). The protocols used to replace the gene *metA* of *E. coli* by the gene *metA* of *C. glutamicum*, or modify the sequence of *metA* of *E. coli* to obtain a homoserine O-acetyltransferase activity in place of a homoserine O-succinyltransferase activity are known to those skilled in the art.

25 Similarly, it is possible to replace or modify the gene *metA* of *C. glutamicum*, coding for a homoserine O-acetyltransferase activity, so as to obtain a homoserine O-succinyltransferase activity.

All the modifications stated above can be carried out directly on the strain subjected to selection pressure when the method according to the invention is
30 implemented. Alternatively, it is preferable to implement the screening process according to the invention on a strain that presents only a limited number of modifications, obtain a strain presenting a 'methionine synthase' activity in the

presence of the compound of formula (II), in particular methyl mercaptan, and carry out other modifications as described, so as to enhance the bypass of the normal methionine synthesis pathway.

Those skilled in the art know the protocols used to modify the genetic characters of micro-organisms. The over-expression of a gene can be achieved by changing a promoter of this gene *in situ* for a strong or inducible promoter. Alternatively, a replicative plasmid (single-copy or multicopy) can be introduced into the cell, in which the gene to be over-expressed is under the control of an appropriate promoter.

10 The inactivation of a gene is preferably carried out by homologous recombination. The protocol principle is briefly as follows: a linear fragment is introduced into the cell. This fragment is obtained *in vitro*, and it comprises the two regions flanking the gene, and at least one selected gene between these two regions (generally a gene for antibiotic resistance). This linear fragment thus presents an inactivated gene. The cells that have undergone a recombination event and have integrated the fragment introduced are selected by plating on a selective medium. The cells that have undergone a double recombination event are then selected, in which the native gene has been replaced by the inactivated gene. This protocol can be improved by using systems of positive and negative selection to speed up the detection of double recombination events.

In a preferred embodiment, the bacterial strain is a strain of *E. coli*.

In another embodiment, the bacterial strain is a strain of *Corynebacterium*, in particular *C. glutamicum*.

In a preferred embodiment of the invention the bacterial strain is the strain *E. coli* K183 registered at the CNCM (French IDA) on 2 April 2003 under the number I-3005. This strain possesses a gene expressing a modified cystathionine- γ -synthase that presents the mutation E325A described previously, and an inactivation of the gene *metE*.

30 The strains of micro-organisms according to the invention possess a cystathionine- γ -synthase enzyme and (or) an acylhomoserine sulphydrylase enzyme. They are preferably selected and improved by a method of screening and evolution, which is also an object of the invention. The strains according to the invention can

also be genetically modified (that is, they can present an inactivation, a mutation and (or) an over-activation of at least one endogenous gene). This modification can be made before or after the implementation of the screening process.

To accelerate the selection and directed evolution of the strains for the production of methionine in the presence of the compound of formula (II), in particular of methyl mercaptan, the operations described below can be performed. The process is described for methyl mercaptan, but those skilled in the art will know how to adapt it to any other compound of formula (II), in particular H_2S .

a. Coupling of the biosynthesis of the substance of interest with the growth of the micro-organism in such a way that the production of that substance becomes necessary for the growth of the micro-organism.

One option is to disrupt the *metE* gene coding for methionine-synthase, which produces methionine from homocysteine. The strain then becomes auxotrophic for methionine.

To survive in a minimum medium containing a simple carbon source and methyl mercaptan or sodium methyl mercaptide, the micro-organism has therefore to optimise the synthesis pathway of L-methionine from O-acyl-L-homoserine and methyl mercaptan or sodium methyl mercaptide. Computer modelling shows that in these conditions it is possible to double theoretical methionine yields (Table 1).

| Biomass yields $Y_{X/S}$ (g/g) | Methionine yields ^a $Y_{P/S}$ (g/g) | Methionine yields ^b $Y_{P/S}$ (g/g) |
|-----------------------------------|--|--|
| 0 | 0.36 | 0.74 |
| 0.11 | 0.30 | 0.62 |
| 0.28 | 0.21 | 0.42 |
| 0.44 | 0.12 | 0.24 |
| 0.61 | 0 | 0 |

Table 1: Maximum theoretical yields for the production of methionine (mass of methionine/mass of glucose) by *E. coli* for fermentation on glucose (a) and for fermentation on glucose and methyl mercaptan (b) with a constant biomass yield (continuous cultures).

However, when a 2-amino-4-(alkylmercapto)butyric acid other than L-methionine is sought, the medium has to be supplemented with methionine to enable the micro-organism to grow.

5 b. Suppressing regulation, in particular negative feedback at either enzyme or gene level, so that the main biosynthesis pathway is potentiated.

The gene *metJ*, which codes for a repressor gene, can thus be suppressed. In addition, it has been shown that homoserine transsuccinylase, coded for by the gene *metA*, is down-regulated by methionine and S-adenosylmethionine (Taylor et al.,
10 1966, J. Biol. Chem., **241**: 3641-3642). It is therefore desirable to replace this enzyme by one that is insensitive to this negative feedback (Chater et al, 1970, J. Gen. Microbiol. **63**: 121-131).

A further object of the invention is a screening and identification test by which it is possible to obtain a micro-organism producing a 2-amino-4-
15 (alkylmercapto)butyric acid, in particular L-methionine, by metabolising an alkyl mercaptan or H₂S, in particular methyl mercaptan.

Thus the present invention makes it possible to identify the strains that possess mutations in their genome such that the strain is able to assimilate a compound of formula (II), and produce the amino acid of formula (I). These
20 modifications thus induce an increase in the 'methionine synthase' activity of that strain. It is thus possible to accelerate the production of the strain producing methionine autonomously from a simple carbon source and a compound of formula (II).

A further object of the invention is thus a method of screening of an initial
25 bacterial strain, which can be a genetically modified bacterial strain, that possesses a gene coding for a cystathionine-γ-synthase enzyme or acylhomoserine sulphydrylase enzyme, in order to obtain a genetically modified bacterial strain producing an amino acid of formula (I), in particular L-methionine, and presenting a modification to the gene of that enzyme that induces a modification to the 'methionine synthase' activity
30 in the presence of a sulfur-containing compound of formula (II), whereby the bacterial strain is subjected to a selection pressure in the presence of a compound of formula (II), so as to direct an evolution of the gene coding for the cystathionine-γ-

synthase enzyme or for the acylhomoserine sulfhydrylase enzyme in that bacterial strain, which evolution consists of a mutation that enables it to carry out a preferential direct conversion of the substrate of general formula (III) into an amino acid of general formula (I) or into homocysteine, of formula (IV).

5 The initial bacterial strain can present an inactivation and (or) an over-activation, in particular by the insertion of a strong constitutive promoter, of at least one endogenous gene.

 The invention also concerns a bacterial strain presenting a modification to the gene of the cystathionine- γ -synthase enzyme and (or) to the gene of the
10 acylhomoserine sulfhydrylase enzyme, inducing an increased 'methionine synthase' activity of that enzyme in the presence of a compound of formula (II), in particular methyl mercaptan. Such a strain can also present at least one other genetic modification (inactivation, mutation or over-expression of an endogenous gene), as described above.

15 The strain according to the invention can preferably be obtained by a method according to the invention, and in particular is obtained by the method according to the invention.

 The invention also concerns a method for the preparation of a 2-amino-4(alkylmercapto)butyric acid of formula (I) defined above, whereby a micro-
20 organism with a modified 'methionine synthase' activity as defined above is grown in the presence of a sulfur-containing compound of general formula (II) in an appropriate medium containing a simple carbon source as defined above. The amino acid of formula (I) is preferably methionine, more preferably L-methionine, and the sulfur-containing compound of general formula (II) is methyl mercaptan or H_2S .

25 According to the invention, the terms 'culture' and 'fermentation' are used interchangeably to designate the growth of bacteria in an appropriate culture medium containing a simple carbon source.

 The culture conditions for micro-organisms according to the invention (fermentation) can be set by those skilled in the art. In particular, bacteria are grown
30 at a temperature between 20°C and 55°C, preferably between 25°C and 40°C, and in particular at about 30°C for *C. glutamicum* and about 37°C for *E. coli*.

The fermentation is carried out in fermenters with a mineral culture medium of known set composition adapted to the bacteria used, containing at least one simple carbon source together with the sulfur-containing compound of formula (II).

In particular, the mineral medium for *E. coli* can be identical or similar in composition to an M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA* 32:120-128), or to an M63 medium (Miller, 1992; *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), or to a medium such as defined by Schaefer *et al.* (1999, *Anal. Biochem.* 270: 88-96).

In the same way, the mineral culture medium for *C. glutamicum* can be identical or similar in composition to BMCG medium (Liebl *et al.*, 1989, *Appl. Microbiol. Biotechnol.* 32: 205-210), or to a medium such as defined Riedel *et al.* (2001, *J. Mol. Microbiol. Biotechnol.* 3: 573-583).

The media can be supplemented to compensate for any auxotrophy. They contain a concentration of simple carbon that is adapted to the culture and production mode, and a sulfur-containing compound of formula (II) at a concentration adapted to the evolution of the strain and the mode of production selected.

After fermentation, the amino acid of formula (I) is recovered by the usual methods and if necessary purified.

The methods for recovery and purification of amino acids of formula (I) in culture media are well-known to those skilled in the art.

The present invention also concerns a method for the preparation of a 2-amino-4(alkylmercapto)butyric acid of formula (I) defined above, whereby a substrate of general formula (III)



where R'' represents an acyl group, preferably either a succinyl or an acetyl group,

reacts with an enzyme with modified 'methionine synthase' activity as described above in an appropriate reaction medium containing a sulfur-containing compound of formula (II) defined above.

The appropriate reaction medium is a usual enzyme reaction medium well known to those skilled in the art, in particular an aqueous medium in which the

substrates and the enzyme are dissolved or suspended. The conditions of implementation of the reaction are well known to those skilled in the art, in particular those required to prevent any substantial denaturing of the enzyme.

In a particular embodiment of the invention, the enzyme with
5 modified 'methionine synthase' activity is present in an inactivated bacterium or in a cell extract.

In another particular embodiment of the invention, the enzyme with modified 'methionine synthase' activity is a purified enzyme.

D.II. The cysteine biosynthesis pathway

10 The present invention also relates to the field of bioconversion and the production of amino acids by fermentation of microorganisms. It relates also to a screening and directed evolution method allowing the identification of strain of microorganisms, possibly genetically modified, possessing an enzyme with "modified cysteine synthase" activity, in particular a modified O-acyl-L-homoserine
15 sulfhydrylase. The corresponding strain produces L-cysteine or an amino acid derivative by metabolism of a simple carbon source. The invention also concerns the strain of a microorganism and the process for the preparation of L-cysteine, or an amino acid derivative, by culturing the said strain of a microorganism.

Cysteine can be produced using various means and different sources. Sun-
20 Orient Chemical Co., Ltd extracts L-cystine from hydrolyzed hair in the presence of HCl. L-cystine is then converted into cysteine monohydrochloride using an electrolytic process.

DL-cysteine monohydrochloride can be produced by the Strecker process (synthesis of L-cystine in the presence of ammonium, HCN and mercaptaldehyde).

25 The Bucherer-Berg reaction, in which chloracetaldehyde, HCN, ammoniumbicarbonate and sodiumsulfide are mixed, allows also the production of L-cysteine. La L-cysteine is crystallized as monohydrochloride in a solution of hydrochloric acid.

The enzymatic production of cysteine or the bioconversion using tryptophane
30 synthase to catalyze the reaction between an alanine substituted in the β -position and a sulfide was described in the patent applications GB2 174 390 and EP0 272 365. Similarly the production of cysteine by fermentation of microorganisms has been

described in the patent applications EP 0 885 962 and WO01/27 307 and WO97/15 673 that describe, respectively, the optimization of the excretion of the synthesized cysteine by the microorganisms, the overexpression of the gene *cysB* in order to optimize the production of H₂S and a serine acetyltransferase insensitive to the feed-back inhibition by cysteine.

The invention can also be applied, for example, to the cysteine biosynthesis pathway (Figure 4) providing strains of evolved microorganisms, in particular bacteria, e.g., *E. coli* and *Corynebacteria*, that produce 2-amino-4-(alkylmercapto) propionic acids with the general formula (V)



where R is defined above as the moiety of the general formula (I)

by the metabolism of a simple carbon source and a source of sulfur consisting of a compound of general formula (II):



defined above

which strains present at least one gene coding for a mutated enzyme with an 'evolved cysteine synthase' activity.

According to the invention a 'physiologically acceptable salt' of the compound of general formula (I) is one that does not effect the metabolism or ability to grow of the microorganism strain according to the invention, in particular the salts of alkali metals such as sodium.

In a preferred embodiment of the invention, R is a straight-chain or branched-chain alkyl radical containing 1 to 4 atoms of carbon, selected in particular from among the methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl and t-butyl groups. R is preferentially a methyl radical.

The 2-amino-4-(alkylmercapto)propionic acid of general formula (V) obtained is preferably L-cysteine.

According to the invention an enzyme with evolved 'cysteine synthase' activity is any mutated enzyme involved in the biosynthesis of the amino acid of general formula (V), in particular L-cysteine, the essential activity of which is to carry out the direct conversion of an acetylserine, preferably O-acetyl-L-serine, into an amino acid of general formula (V) in the presence of a sulfur compound of

general formula (II), whereas the essential activity of the initial non-mutated enzyme was not a 'cysteine synthase' activity. The enzymes naturally involved in the biosynthesis of cysteine by the direct conversion of O-acetyl-L-serine (acetylserine) into L-cysteine in the presence of hydrogen sulfide (H₂S), such as cysteine synthases
 5 A and B, coded for by genes *cysK* or *cysM*, respectively, are therefore excluded from this definition.

The 'initial' non-mutated enzyme is preferentially an enzyme that catalyzes a sulphydrylation reaction in the presence of H₂S, preferably an enzyme with O-acyl-L-homoserine sulphydrylase activity.

10 The 'initial' enzymes with O-acyl-L-homoserine sulphydrylase activity are advantageously selected from among the O-acyl-L-homoserine sulphydrylases corresponding to PFAM reference PF01053 and COG reference COG2873.

They are preferentially chosen among the following acylhomoserine:

- NP_785969 O-acetylhomoserine (thiol)-lyase, *Lactobacillus plantarum* WCFS1
- 15 AAN68137 O-acetylhomoserine sulphydrylase, *Pseudomonas putida* KT2440
- NP_599886 O-acetylhomoserine sulphydrylase, *Corynebacterium glutamicum* ATCC 13032
- NP_712243 acetylhomoserine sulphydrylase, *Leptospira interrogans* serovar lai str. 56601
- 20 BAC46370 O-succinylhomoserine sulphydrylase, *Bradyrhizobium japonicum* USDA110
- AAO57279 O-succinylhomoserine sulphydrylase, *Pseudomonas syringae* pv. tomato str. DC3000
- NP_284520 O-succinylhomoserine sulphydrylase [*Neisseria meningitidis* Z2491
- 25 AAA83435 O-succinylhomoserine sulphydrylase (*P. aeruginosa*)

With higher preference the O-acyl-L-homoserine sulphydrylase is selected from among the O-acyl-L-homoserine sulphydrylases coded for by the gene *metY* of *Corynebacterium*, in particular the gene *metY* of *C. glutamicum* (Genbank
 30 AF220150), and the homologous enzymes presenting the same O-acyl-L-homoserine sulphydrylase activity, and at least 80% sequence homology with O-acyl-L-homoserine sulphydrylase coded for by the gene *metY* of *C. glutamicum* (Genbank

AF220150), preferably at least 85% homology, and more preferably at least 90 % homology.

The invention is in particular based on the fact that it is possible to obtain, in a controlled manner, a modification of the substrate specificity of the enzyme acyl-
5 homoserine sulfhydrylase so that it uses acetylserine preferentially. Consequently the invention is based on the fact that it is possible to modify, in a controlled manner, the substrate specificity of the unmodified enzyme in order to evolve from an acylhomoserine sulfhydrylase activity to a cysteine synthase activity.

In a preferred embodiment of the invention, the strains of unmodified
10 microorganisms do not naturally possess O-acetyl-L-homoserine sulfhydrylase activity or do not possess a gene homologous to the gene *metY* that codes for that enzyme.

The strains modified according to the invention are genetically modified by the inactivation, mutation and/or overactivation of at least one endogenous gene in order to permit the evolution of a new metabolic pathway. In particular, the strains of
15 microorganisms according to the invention are genetically modified to suppress the genes *cysK* and/or *cysM* coding for the proteins respectively bearing the cysteine synthase A, and cysteine synthase B enzyme activities. The genes *cysK* and *cysM* are preferentially suppressed.

The gene *cysK* (Figure 4) codes for cysteine synthase A (GenBank
20 NP_416909) and the gene *cysM* codes for cysteine synthase B (GenBank NP_416916). The effect of inactivating these genes closes the cysteine biosynthesis pathways and so makes the strain auxotrophic for cysteine.

A gene is then introduced into the modified bacteria that codes for an enzyme catalyzing a sulfhydrylation reaction in the presence of H₂S, as defined previously,
25 other than those enzymes the main activity of which is a cysteine synthase activity (also called O-acetylserine sulfhydrylase), in particular a gene coding for an O-acetyl-L-homoserine sulfhydrylase, such as the gene *metY* of *Corynebacterium*, and in particular the gene *metY* of *C. glutamicum* (Genbank AF220150).

The gene coding for an enzyme catalyzing a sulfhydrylation reaction in the
30 presence of H₂S can be introduced into the bacterium to be modified by the usual methods available to those skilled in the art, either by direct integration or carried by a replicative plasmid.

The strain modified in this way is preferably selected and improved by a method of screening and evolution, which is also an object of this invention, and which makes it possible to cause the acyl-homoserine sulfhydrylase activity to evolve into a cysteine synthase activity to restore the production of cysteine.

5 The transformation of the acyl-homoserine sulfhydrylase activity into an 'evolved cysteine synthase' activity is deemed to be achieved when the genetically modified and evolved bacterial strain (E) has a growth rate at least similar to that of the initial modified strain (M) when grown in a minimal medium in the presence of glucose as a single carbon source. In a particular embodiment the transformation of
10 the acyl-homoserine sulfhydrylase activity into the 'evolved cysteine synthase' activity is deemed to be achieved when the cysteine synthase activity carried by the modified O-acyl-L-homoserine sulfhydrylase protein has been improved by 10% relative to its initial activity. In some cases this improvement can be observed by an increased growth rate of bacterium E relative to that of bacterium M. Lastly, this
15 transformation of the acyl-homoserine sulfhydrylase activity into an 'evolved cysteine synthase' activity will be deemed achieved when strain E produces at least as much cysteine as strain M in equivalent culture conditions, in the absence of any initial cysteine content.

The strains according to the invention can also be genetically modified by
20 inactivation, mutation and/or overactivation of at least one endogenous gene, such modification being made before or after the evolution step of the modified strain. In particular according to the invention the strains of microorganisms are genetically modified in order to suppress the enzymatic activities cysteine synthase A, cysteine synthase B and cystathionine- γ -synthase. Preferentially the genes *cysK* and *cysM* are
25 suppressed. The gene *cysK* encodes the cysteine synthase A (GenBank NP_416909) and the gene *cysM* encodes cysteine synthase B (Genbank NP_416916). The inactivation of these genes leads to a suppression of the cysteine biosynthetic pathways and thus renders the strain auxotrophic for cysteine. This allows to select the strains that have modified the acyl-homoserine sulfhydrylase activity into a
30 cysteine synthase activity to restore the production of cysteine.

The gene coding for cystathionine gamma-lyase activity can be deleted, if required.

In one embodiment the bacterial strain can also undergo modification of the serine O-acyltransferase activity carried by the gene *cysE* (Figure 4) to make it insensitive to feedback inhibition by cysteine.

In another embodiment the bacterial strain can also be made to overexpress
5 the gene *cysB* in order to deregulate the H₂S sulfur assimilation pathway.

It can also be advantageous for the production of amino acids of formula (V), preferably L-cysteine, to overexpress the gene coding for acetyl-CoA synthetase, such as the gene *acs* (EC 6.2.1.1), accession number AE000480, at the same time as the gene coding for an enzyme with an 'improved cysteine synthase activity' defined
10 previously (Figure 4).

It can also be advantageous to attenuate or even delete genes coding for an acetate kinase and/or for a phosphotransacetylase, in particular the genes *ack* and *pta* accession numbers AE000318 and AE000319 respectively, and coding respectively for an acetate kinase (EC 2.7.2.1) and a phosphotransacetylase (EC 2.3.1.8). This
15 attenuation/deletion is preferably combined with an overexpression of the gene coding for acetyl-CoA synthetase.

All the modifications stated above can be made on the modified strain before the process of evolution of the gene *metY* or on the evolved strain, that is after the evolution of the gene *metY*, but not for deletions of *cysK* and/or *cysM*, which have to
20 be carried out before the process of growth and evolution of the modified strain.

The invention thus further concerns a method for the preparation of a bacterial strain with an 'evolved cysteine synthase activity' as defined previously, which method comprises a step consisting in growing, in an appropriate culture medium containing a simple carbon source, a bacterial strain possessing an enzyme
25 catalyzing a sulphydrylation reaction in the presence of H₂S of which the essential activity is not a 'cysteine synthase' activity as defined previously, in order to cause an evolution of the gene coding for that enzyme in that bacterial strain into a gene coding for an 'evolved cysteine synthase' activity.

In order to accelerate the selection and directed evolution of a gene coding for
30 an enzyme catalyzing a sulphydrylation reaction in the presence of H₂S, the following steps can be carried out:

a. Coupling of cysteine biosynthesis with the growth of the microorganism in a way that the production of cysteine is required for good growth of the microorganism.

For this reason the genes *cysK*, *cysM* and possibly *metB* and possibly the
 5 gene coding for a cystathionine gamma lyase (e.g. the gene *yrhB* in *B. subtilis*) may be deleted, in order to suppress all natural cysteine synthesis pathways. In this way the strain becomes auxotroph for cysteine.

In order to live in minimal medium containing a simple carbon source, the microorganism has to optimize the cysteine synthase activity of the O-acyl-
 10 homoserine sulfhydrylase, so that it can reestablish the synthetic pathway of cysteine starting with acetylserine and H₂S. When *metB* is deleted, it can be necessary to supplement the medium with methionine.

b. Suppressing the regulations, notably the feed-back inhibition on the enzymatic level or genetic level in order to potentiate the major biosynthetic
 15 pathway.

The *cysB* gene encoding the activator protein of the assimilatory sulfur pathway can be overexpressed (WO01/27307) allowing the optimization of H₂S production. Furthermore it has been shown that serine acetyltransferase encoded by the *cysE* gene, is feed-back inhibited by cysteine. Therefore it is desirable to replace
 20 this enzyme by an enzyme that is not sensitive to feed-back inhibition (WO97/15673; WO 02/061106) or alternatively overexpressing the enzyme (WO 02/29029).

The invention further concerns a method for the preparation of cysteine, in which a microorganism with an 'evolved cysteine synthase' activity as defined previously is grown in an appropriate culture medium containing a simple carbon
 25 source as defined previously.

The definition of the fermentation conditions belongs to those skilled in the art. The fermentation is conducted in fermenters with an inorganic growth medium of known set composition adapted according to the bacteria used, containing at least one simple carbon source.

30 The media can be supplemented to compensate for the auxotrophies other than that caused by the deletion of the genes *cysK* and/or *cysM*; they contain a simple source of carbon at a concentration adapted according to the mode of growth and

production, and a sulfur compound of formula (II) at a concentration adapted according to the evolution of the strain and to the desired mode of production.

After fermentation, the cysteine is recovered by the usual methods and purified if necessary.

5 The methods of recovery and purification of cysteine in culture media are well known to those skilled in the art.

This invention further concerns a method for the preparation of an amino acid of general formula (V) as defined previously, characterized in that acetylserine is made to react with an enzyme with an 'evolved cysteine synthase' activity as defined
10 previously, in an appropriate reaction medium containing a sulfur compound of general formula (II) defined previously.

The appropriate reaction medium is a usual enzyme reaction medium, well known to those skilled in the art, in particular an aqueous medium in which the substrates and the enzyme are dissolved or suspended. The operating conditions for
15 the reaction are well known to those skilled in the art, in particular those required to prevent substantial denaturing of the enzyme.

In a particular embodiment of the invention, the enzyme with an 'evolved cysteine synthase' activity is present in an inactivated bacterium or in a cell extract.

D.III. The evolution of NADPH-dependent pathways.

20 In another preferred embodiment of the invention, a NADPH-dependent pathway can be caused to evolve. To this end the initial bacterial strain must be modified in such a way that the rate of production of NADPH is greater than its rate of oxidation to NADP⁺ (also designated NADP), which will prevent growth of the bacteria on the defined medium selected to implement the method.

25 This invention concerns strains of microorganisms modified to permit the evolution of metabolic pathways that consume NADPH. The strains according to the invention can be used in biotransformation processes that consume NADPH.

This invention further concerns a method for the preparation of chemical entities by biotransformation involving the growth in an appropriate medium of a
30 strain according to the invention, which strain also possesses the genetic features necessary for the preparation of such chemical entities.

Biotransformation processes have been developed to permit the production of large quantities of chemical entities at low cost, while at the same time putting to good use various industrial or agricultural by-products.

There are two main approaches to producing chemical entities of interest by
5 *in vivo* biotransformation:

- first, fermentation, which allows the production of chemical entities from a simple carbon source (*e.g.*, WO0102547, which describes the production of lysine by fermentation of *C. glutamicum* in the presence of glucose),
- second, bioconversion by a microorganism of a given substrate different from the
10 simple carbon source, which is used solely to produce the necessary biomass, for the production of a chemical entity of interest (*e.g.*, WO0012745, which describes the production of R-piperidine derivatives, and WO0068397, which describes the production of Tagatose).

The improvement of a biotransformation process can involve various factors
15 such as temperature, oxygenation, the composition of the medium, the recovery process, etc. It is also possible to modify the microorganism in such a way that the production and/or excretion of the chemical entity of interest is increased.

For fermentation, efforts may be directed, for example, to optimizing the biosynthesis pathway by, for example, modifying the regulation of genes or by
20 modifying genes to modify enzyme characteristics or by optimizing the regeneration of co-substrates.

For bioconversion, efforts may be directed to improving the kinetic characteristics of enzymes, to reducing the formation of co-products and to optimizing the regeneration of co-substrates involved in the bioconversion step or
25 steps.

Among the co-substrates involved in biotransformations, NADPH is important, in particular for the production of amino acids (*e.g.*, arginine, proline, isoleucine, methionine or lysine), vitamins (*e.g.*, pantothenate, phyloquinone or tocopherol), aromatic compounds (*e.g.*, WO9401564), or other chemicals with high
30 added value.

This invention concerns strains of microorganisms modified to cause the evolution of enzymes or metabolic pathways that consume NADPH.

For the creation of such microorganisms, the inventors chose modifications that increase the rate of production of NADPH and reduce its rate of oxidation to NADP⁺, which modified microorganisms are then used to cause the evolution of enzymes or metabolic pathways that consume NADPH. These bacteria can also be
 5 used judiciously in biotransformation processes for the production of chemical entities derived from NADPH-dependent synthesis pathways.

The optimization of NADPH is described below for *E. coli*. The same principle can be applied in a similar way to all microorganisms grown in aerobic conditions.

10 The strains modified according to the invention have undergone the deletion of the gene *udhA* and/or the gene *gor*. In a preferred embodiment of the invention, the genes *udhA* and *gor* are both deleted.

In a particular embodiment of the invention the strain modified according to the invention has also undergone the deletion of a gene selected from among *pgi* or
 15 *pfkA* and or *pfkB*.

The above genes are well known to those skilled in the art and are described in the scientific literature, in particular for *E. coli*:

Genes and references in *E. coli*:

udhA: X66026 soluble pyridine transhydrogenase;
 20 *gor*: L02312 quinone oxidoreductase;
pgi: X15196 phosphoglucose isomerase (EC 5.3.1.9);
pfkA: X02519 phosphofructokinase-1;
pfkB: K02500 phosphofructokinase-2.

A further object of this invention is an evolved microorganism modified for
 25 the production of NADPH as described above and below, which also possesses one or more genes coding for NADPH-dependent enzymes involved in the biotransformation of a chemical entity of interest, which enzymes it is intended to cause to evolve, together with one or more selection marker genes.

These genes can be native to the modified strain according to the invention or
 30 be introduced into the optimized strain according to the invention by transformation with an appropriate vector, either by integration in the genome of the microorganism, or by a replicative vector, which vector possesses one or more genes coding for the

enzymes involved in the bioconversion of the said chemical entity of interest or the said selection markers.

These genes contain a nucleic acid sequence coding for an enzyme involved in the biotransformation of the chemical entity of interest and/or for a selection
5 marker, the coding sequence being attached to efficient promoter sequences in the prokaryotic and/or eukaryotic cell selected for the biotransformation. The vector (or plasmid) can be a shuttle vector between *E. coli* and another microorganism.

This invention further concerns a method for the preparation of modified strains according to the invention as defined above and below, in which the genes
10 *udhA* and/or *qor*, and if necessary the genes *pgi* or *pfkA* and/or *pfkB* are deleted.

In a particular embodiment of the invention the method of preparation of the strains also includes the transformation of the modified strains with at least one appropriate vector with one or more genes coding for one or more NADPH-consuming enzymes involved in the biotransformation of a chemical entity of
15 interest, and one or more selection marker genes. The vector allows either the replication of the genes or their integration in the chromosome of the modified bacterium. The transformation of the strain by the vector defined above can be carried out on the modified strain or before the modification of the strain according to the invention.

20 The strain modified for the production of NADPH is obtained by molecular biology methods.

A further aspect of the invention concerns the use of these modified strains according to the invention to cause the evolution of NADPH-dependent enzymes to improve their kinetic characteristics, to broaden or to narrow their substrate
25 specificity, and ultimately to create a new metabolic pathway and/or to improve biotransformation yields. A further aspect of the invention concerns the use of the modified strain or the evolved strain to carry out biotransformation reactions that consume NADPH with biotransformation yields greater than those obtained with an unmodified and/or non-evolved strain.

30 The invention further concerns a method for the production of a chemical entity of interest synthesized by a NADPH-consuming process, characterized in that it comprises the following steps:

- a) Culture of evolved microorganisms according to the invention in an appropriate culture medium that favors their growth and that contains the substances necessary to carry out the biotransformation by fermentation or bioconversion, except for NADPH.
- 5 b) Extraction of the chemical entity of interest from the medium and, if necessary, its purification.

The chemical entity of interest is preferably selected from among cysteine, methionine, 3-hydroxypropionate, hydrocortisone, xylitol and glycerol.

For a bioconversion reaction the method also includes the addition to the
10 appropriate culture medium of the substrate to be converted.

The culture medium of step b) of the method according to the invention defined above contains at least one assimilatable carbohydrate chosen from among the different assimilatable sugars such as glucose, galactose, sucrose, lactose, or molasses, or by-products of these sugars. An especially preferred simple carbon
15 source is glucose. Another preferred simple carbon source is sucrose. The culture medium can also contain one or more substances (e.g., amino acids, vitamins, mineral salts, etc.) that favor the production of the chemical entity of interest.

The examples given below illustrate the invention but do not restrict its scope.

E. Description of figures

20 Figure 1: Synthesis of methionine from homoserine in bacteria.

Figure 2: Scheme for the synthesis of methionine according to the invention applied in *E. coli*; an equivalent strategy is transposable to many microorganisms including *C. glutamicum*. The *metB** or *metY*** strategies require the use of a strain that is initially at least $\Delta(\text{metE})$, while the *metB*** or *metY** strategies require the use
25 of a strain that is initially at least $\Delta(\text{metC})$.

Legend

MetA: homoserine succinyltransferase; can be replaced by an isoform that is insensitive to feedback inhibition by methionine, or possibly by a homoserine acetyltransferase isoform that is insensitive to feedback inhibition by methionine.

30 MetB: cystathionine γ -synthase

MetB*: cystathionine γ -synthase evolved into 'methionine synthase'

MetB^{**}: cystathionine γ -synthase evolved into homocysteine synthase

MetY^{*}: O-acetyl-homoserine (de *C. glutamicum*) evolved into homocysteine synthase

MetY^{**}: O-acetyl-homoserine (de *C. glutamicum*) evolved into 'methionine synthase'

The central pathway represents the natural synthetic pathway of methionine in *E. coli*. The other pathways indicated correspond to the methods according to the invention.

Figure 3 representation of a continuous fermentation mechanism for the directed selection of strains according to the invention. We use for example the technology "Fedbatch-Pro" by the company DASGIP. A modular system is controlled by a computer allowing the parallel fermentation of microorganisms with a control of feeding of medium, pH and pO₂.

Figure 4: Synthesis of cysteine from serine in bacteria.

Figure 5: Strategy to achieve the synthesis of cysteine from O-acetyl-L-serine. The red arrow corresponds to the cysteine synthase activity of the enzyme coded for by the evolved gene *metY* according to the invention (*metY*^{*}).

Figure 6: Comparison of ¹³C-NMR spectra, corresponding to the 5-carbon of methionine, obtained by HSQC on a hydrolysate of the wild strain (top) or the optimized K1a-F strain (bottom). It can be observed that the 5-carbon of the strain K1a-F is not labeled by carbon 13, confirming that it originates from sodium methylmercaptide.

Figure 7: Alignment of non-modified sequences of cystathionine- γ -synthases obtained with the algorithm MULTIALIN (<http://prodes.toulouse.inra.fr/multalin/multalin.html>).

Figure 8: Alignment of non-modified sequences of acylhomoserine sulphydrylases obtained with the algorithm MULTIALIN.

Figure 9: Growth rate evolution of the initial *E. coli* strain $\Delta(\text{metE})$ during the process of directed selection in batch culture. Abscissa: number of subcultures; arrow: population K144; deduced μ : values obtained starting at OD values of 2-3; calculated μ : values obtained with more than 4 values of DO.

Figure 10: Growth kinetics of the population of *E. coli* $\Delta(\text{metC})$ after initial seeding (Culture 1) and after tenth reseeding (Reseeding 10).

Figure 11: Execution of PCR on the modified strain of *E. coli* $\Delta\text{metJ}::\text{Cm}$ using starters DmetJBF and MetJR. The 5' end of the starter DmetJBF is also able to
5 hybridise a sequence located in the 3' part of the gene *metL*.

Figure 12: Strains obtained after homologous recombination with the PCR-amplified fragment (see Figure 11); it is possible to have two different homologous recombination events, each occurring with the same probability. In the first case, a strain *E. coli* $\Delta\text{metJ}::\text{Cm}$ is recreated, while in the second case the strain *E. coli*
10 [ΔmetJ , $\Delta\text{metJ}::\text{Cm}$] is created by replacement of the promoter of the operon *metBLF* before *metL*.

Figure 13: Time course of the growth rate evolution of the strain *E. coli* [$\Delta(\text{metBJ}$, *metC*) pTrc-*metY*] on a defined medium (MML8) containing glucose as sole carbon source.

15 F. Examples

F.I. The methionine biosynthesis pathway

A first application (Example F.I.1.) of the invention to the metabolic engineering of the biosynthesis pathway of methionine comprises the following steps:

- a) Deletion of the gene *metE* in the initial strain of *E. coli*; the modified strain
20 obtained is therefore auxotrophic for methionine. The initial strain is able to grow on a minimal medium (MM) containing no methionine, S-adenosylmethionine, homocysteine or cystathionine, whereas the modified strain has lost that ability.
- b) Culture of the above modified strain on the same minimal medium (MM) to
25 which sodium methylmercaptide (co-substrate) has been added to cause the evolution of an endogenous enzyme activity into a methionine-synthase activity to compensate for the initially deleted enzyme activity (MetE).
- c) Selection of an evolved strain with a new methionine-synthase activity in the presence of sodium methylmercaptide, the strain being characterized.

- d) Isolation of the evolved gene coding for the protein possessing an evolved enzyme activity, in this case cystathionine-γ-synthase with an improved methionine-synthase activity.

A second application (Example F.I.2.) of the invention to the metabolic engineering of the biosynthesis pathway of methionine comprises the following steps:

- a) Deletion of the gene *metC* in the initial strain of *E. coli*; the modified strain obtained is thus auxotrophic for methionine. The initial strain is able to grow on a minimal medium (MM) containing no methionine, S-adenosylmethionine, homocysteine or cystathionine, whereas the modified strain has lost that ability.
- b) Culture of the above modified strain on the same minimal medium (MM) in the absence of any co-substrate in order to cause the evolution of an endogenous enzyme activity into a homocysteine-synthase activity to compensate for the initially deleted enzyme activity (MetC).

A third application (Example F.I.3.) of the invention to the evolution of the biosynthesis pathway of methionine comprises the following steps:

- a) Deletion of the genes *metC*, *metB*, and *metJ* in the initial strain of *E. coli*; the modified strain obtained is thus auxotrophic for methionine. The initial strain is able to grow on a minimal medium (MM) containing no methionine, S-adenosylmethionine, homocysteine or cystathionine, whereas the modified strain has lost that ability.
- a1) Introduction of the gene *metY*, a heterologous gene from *C. glutamicum*. This gene is to evolve from an acetylhomoserine sulfhydrylase activity into a methionine-synthase activity.
- b) Culture of the modified strain *E. coli* [*metY* Δ (*metB*, *metC*, *metJ*)] on the same minimal medium (MM) to which is added sodium methylmercaptide (co-substrate) to cause the evolution of an endogenous enzyme activity into a methionine-synthase activity to compensate for the initially deleted enzyme activities (MetB, MetC).
- c) Selection of an evolved strain possessing a new methionine-synthase activity in the presence of sodium methylmercaptide.

Example F.I.1 Preparation of an evolved strain possessing a methionine-synthase activity in the presence of sodium methylmercaptide

a) Construction of the modified strain *E. coli* Δ (*metE*)

The inactivation of the gene *metE* is achieved by inserting a chloramphenicol
 5 resistance gene cassette and at the same time deleting most of the gene concerned.
 The method used is described by Datsenko, K.A., Wanner, B.L. (2000) One-step
 inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products.
Proc. Natl. Acad. Sci. USA 97: 6640-6645.

Two oligonucleotides were used to implement this strategy:

10 - DmetER with 100 bases (SEQ ID NO 1):

tacccccgacgcaagttctgcgccgctgcaccatgttcgccagtgccgcgcgggtttctggccagccgcgc
 gttttcagCATATGAATATCCTCCTTAG

with:

- a region (lower case) homologous to the sequence (4012903 to 4012824) of
 15 the gene *metE* (sequence 4010643 to 4012904, the reference sequence on the website
<http://genolist.pasteur.fr/Colibri/>);

- a region (upper case) for the amplification of the chloramphenicol resistance
 cassette of the plasmid pKD3 (Datsenko, K.A., Wanner, B.L. (2000) One-step
 inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products.
 20 *Proc. Natl. Acad. Sci. USA* 97: 6640-6645).

- DmetEF de 100 bases (SEQ ID NO 2):

tgacaatattgaatcacaccctcggttcctcgcggttgccctgcgtcgcgagctgaaaaagcgcaagaaagt
 tattggTGTAGGCTGGAGCTGCTTCG

with:

25 - a region (lower case) homologous to the sequence (4010644 to 4010723) of
 the gene *metE*;

- a region (upper case) for the amplification of the chloramphenicol resistance
 cassette carried by the plasmid pKD3.

The oligonucleotides DmetER and DmetEF are used to amplify the
 30 chloramphenicol resistance cassette from the plasmid pKD3. The PCR product
 obtained is then introduced by electroporation into the strain MG1655 (pKD46) in
 which the enzyme Red recombinase expressed permits the homologous

recombination. The chloramphenicol-resistant transformants are then selected and the insertion of the resistance cassette is verified by PCR analysis with the oligonucleotides metER and metEF.

MetER (SEQ ID NO 3): gggttaagcagtatggtgggaagaagtcgc (homologous to the
5 sequence from 4012978 to 4012949).

MetEF (SEQ ID NO 4): cccggggatgaataaacttgccgccttccc (homologous to the sequence from 4010567 to 4010596).

The chloramphenicol resistance cassette can then be eliminated. The plasmid pCP20 carrying the FLP recombinase acting at the FRT sites of the chloramphenicol
10 resistance cassette, is then introduced into the recombinant strains by electroporation. After a series of cultures at 42°C, the loss of the chloramphenicol resistance cassette is verified by a PCR analysis with the same oligonucleotides as used previously.

b) Culture and evolution of the $\Delta(metE)$ modified strain in the presence of sodium methylmercaptide as co-substrate

15 To optimize *E. coli* for the production of methionine from methylmercaptan, a controlled selection is carried out in flasks.

The *E. coli* strain made auxotrophic for methionine by inactivating the *metE* gene (see above) cannot grow unless it can make its own methionine from methylmercaptan.

20 The implementation of this method permits the selection of a strain of *Escherichia coli* of which the cystathionine- γ -synthase (EC 4.2.99.9) has acquired a modified 'methionine-synthase' activity in the presence of methylmercaptan.

The controlled selection is conducted in a hermetically sealed glass flask containing 50 ml of inorganic medium (Schaefer et al., 1999, *Anal. Biochem.* **270**:
25 88-96) in the presence of 33 mM glucose and chloramphenicol at a final concentration of 25 mg/l.

The culture media are seeded with the strain *E. coli* K12 $\Delta metE$ at a defined value of OD_{600nm}. Seeding is carried out with a sufficiently large population of bacteria so that some bacteria potentially possess relevant spontaneous mutations in
30 the gene *metB* enabling assimilation of methylmercaptan. This population is obtained by culture of the strain auxotrophic for methionine on a minimal medium supplemented with methionine.

Three flasks then receive 100 μ l of a 400 mg/l solution of sodium mercaptide, while a fourth flask has no added sodium mercaptide. The cultures are grown with shaking at 37°C, for 6 days, after which time the OD_{600nm} is measured. The results are summarized in Table 2 below.

5

| | Flask 1 | Flask 2 | Flask 3 | Control flask |
|-----------------------------------|---------|---------|---------|---------------|
| OD _{600nm} at $T=0$ | 0.34 | 0.34 | 0.34 | 0.34 |
| OD _{600nm} at $T=6$ days | 0.23 | 1.14 | 0.79 | 0.32 |

Table 2. Measurement of the optical density (OD) of culture media for *E. coli* in the presence (flasks 1-3) or absence (control flask) of sodium mercaptide.

These results show that in flasks 2 and 3 a strain grows that is able to use methylmercaptan to produce the methionine necessary for its growth (increase in optical density).

10

The observed improved “methionine synthase” activity results from a modification of the gene encoding cystathionine- γ -synthase of *E. coli* K12 Δ metE, contained in flask 2 and 3.

The bacterial population of flask 2 can then be used to improve even more the “methionine synthase” activity in the presence of methylmercaptan, using a screening process and improvement by multi-stage fermentation or by starting over the process in flasks as described above.

15

c) Screening and improvement by multi-stage fermentation

To optimize *E. coli* for the production of methionine based on methylmercaptan, a selection is performed.

20

The implementation of this technique allows the selection of an *Escherichia coli* strain in which the cystathionine- γ -synthase (EC 4.2.99.9) has developed an improved “methionine-synthase” activity in the presence of methyl-mercaptan. Alternatively a strain obtained in example 2 can be used.

25

The directed selection is carried out in a continuous multi-stage system (Figure 3).

The first fermenter produces bacteria with a speed close to maximal growth rate. The bacteria are transferred continually from this fermenter to a second

fermenter characterized by a lower dilution rate with a selective screen (here methylmercaptan).

The selection pressure, imposed on the bacteria in the second fermenter, depends on the methylmercaptan concentration. Successive cycles of selection allow
 5 applying stronger and stronger screening conditions to the bacteria by increasing methylmercaptan concentrations.

For each concentration the selected strain in the second fermenter has evolved so that it metabolizes the total amount of methylmercaptan (no residual methylmercaptan in the fermenter).

10 In this case one starts the selection over using fermenter 2 as growth fermenter and fermenter 1 for selection, presenting methylmercaptan in stronger concentration as in the previous step.

Different cycles of selection are performed in order to obtain a strain that rapidly ferments methylmercaptan. The analysis of this strain allows to define the
 15 mutations in the gene cystathionine- γ -synthase.

d) Selection of the evolved strain in the presence of sodium methylmercaptide

The population of *E. coli* $\Delta(\text{metE})$ from flask 2, after undergoing successive reseedings in flasks, yields the population K1a-F.

20 The new population obtained K1a-F is cultured in a minimal medium (Schaefer *et al.*, 1999, *Anal. Biochem.* **270**: 88-96) containing 2.5 g.l⁻¹ of uniformly carbon 13-labeled glucose, and sodium methylmercaptide (200 ppm) with no carbon 13 enrichment. This population is auxotrophic for methionine in the absence of sodium methylmercaptide.

25 After culture, the cells are recovered, washed and hydrolysed with 6N HCl for 24 hours at 107°C. An analysis by 2D NMR is then carried out (HSQC). This analysis shows whether the 5-carbon of methionine comes from L-cysteine produced from the glucose present in the solution (classical pathway), or from the sodium methylmercaptide when the new metabolic pathway according to the invention is
 30 used.

The experiment is conducted in a similar manner with the wild strain *E. coli* K12 (producing methionine from glucose), in the absence of sodium methylmercaptide.

Figure 6 shows two 1D spectra, derived from two separate acquisitions, superimposed for greater clarity. These 1D spectra were extracted from 2D NMR spectra of the HSQC type (correlation between protons and carbon 13). The 2D NMR spectra are obtained on an acid hydrolysate of the bacteria.

The sample analyzed is a total hydrolysate. However, given the sensitivity of the NMR and the acquisition times used, essentially amino acids, sugars, bases and glycerol are detected, each carbon atom (coupled to a proton) of each acid giving an NMR signal.

The 5-carbon of methionine (i.e., the terminal methyl group) presents a chemical shift of about 14.7 ppm. Figure 6 shows the area of the chemical shift centered at 14.7 ppm for the two strains.

It can be seen that the signal of the 5-carbon is strong in the upper spectrum, indicating that the 5-carbon is labeled with carbon 13. Hence this 5-carbon comes from the labeled glucose introduced as a substrate in the culture medium.

In contrast, the same signal is very weak in the lower spectrum (strain K1a-F). This indicates that the 5-carbon is practically unlabeled. Yet the other carbons in the molecule are strongly labeled (results not presented). The unlabeled 5-carbon therefore comes not from glucose, but from methylmercaptan.

It can therefore be concluded that the strain K1a-F produces methionine from succinyl-L-homoserine and sodium methylmercaptide.

The population K1a-F undergoes 14 further successive reseeding cycles in flasks. In this way the population K144 is obtained (Figure 9), which is then spread on minimal medium plates containing glucose as sole carbon source. The inoculated dishes are placed in aerobic conditions in an aerobic jar into which is inserted a tube containing sodium methylmercaptide dissolved in water. The jar is then placed in an incubator at 37°C. As the boiling point of methylmercaptan is 5°C, the atmosphere in the jar becomes enriched in methylmercaptan. After 4 days, clones appear in the dishes; these are bacteria able to produce methionine in the presence of

methylmercaptan. Ten clones are isolated, including the clone K176. The clone K176 is grown in liquid culture and glycerol stocks are prepared, numbered K183.

For the clone K183 and the initial strain *E. coli* K12 $\Delta(\text{metE})$, the sequence of the genes *metJ* and *metB* (SEQ ID No.5) is determined. The sequence obtained for evolved *metB*, designated *metB** (SEQ ID No.7) reveals the presence of an alanine unit at position 325 (SEQ ID No.8) in place of a glutamate (SEQ ID No.6). The gene *metJ* shows no mutation. This strain is registered at the CNCM on April 2 2003, under the number I-3005.

• Characterization of the clone K183

The clone K183 is grown in flasks in a minimal medium with glucose and sodium methylmercaptide as sole carbon source. In parallel, a culture is carried out under identical conditions with wild strain *E. coli* K12. The consumption of glucose per unit of biomass is found to be twice that of a wild strain of *E. coli* (MBM01). This over-consumption is probably partly due to acetate production.

| Strain | Biomass yield | Acetate yield |
|--------|---------------|---------------|
| MBM01 | 0.45 | < 0.002 |
| K183 | 0.24 | 0.36 |

Table 3. Comparison of biomass yield of wild strain *E. coli* and evolved clone K183:

Biomass yield expressed as mass of biomass / mass of glucose

Acetate yield expressed as mass of acetate / mass of glucose

The analysis of intracellular and extracellular metabolites of these two cultures shows, in particular:

Intracellularly, an increase in alanine, pyruvate, ketobutyrate and 2-ketoisocaproate, and a decrease in the concentration of tryptophan, norvaline, norleucine, leucine and methionine.

Extracellularly, an accumulation of glutamate, isoleucine, threonine, valine and 2-ketoisocaproate, and a decrease in pyruvate, norleucine, and tryptophan.

• **Characterization of the specific 'methionine synthase' activity of the strains MBM01 and K183 in the presence of methylmercaptan.**

To show the improvement of the methionine-synthase activity in the strain K183 relative to the wild strain (MBM01), enzyme reactions are carried out using cell-free extracts prepared from cultures of the strains K183 and MBM01 carried out

on rich medium (BH1, marketed by DIFCO, with 2.5 g/L of glucose) in the absence of methylmercaptan. The protein extracts are desalted on PD10 and stored on ice.

Reaction conditions and sample treatment

- A solution of sodium methanethiolate diluted 10-fold (100 μ L of 3M solution plus 900 μ L of MilliQ water) is prepared on ice.
- Reaction mixtures of 20 μ L of 500 mM pH 6.5 phosphate buffer, 10 μ L of 2.5 mM pyridoxal phosphate, 16 μ L of 25 mM O-succinylhomoserine, 10 μ L of 0.3 M sodium methanethiolate, and 24 μ L of MilliQ water are prepared on ice.
- The tubes are placed at 37°C (thermomixer under a hood) and the protein extract (20 μ L) added to start the reaction.
- To quench the reaction (0 to 30 minutes), the tubes are placed on ice and 400 μ L of acetone added at -20°C.
- The tubes are left at -20°C for 30 minutes.
- The tubes are opened under the hood for 10 minutes to evaporate off methanethiol and acetone (kept on ice).
- The mixture is centrifuged for 5 minutes at 10,000 g, the supernatant (~100 μ L) decanted off into Eppendorf tubes and diluted to a final volume of 1 ml.

Measurement of the methionine synthase activity by detection of the quantity of succinate released from succinylhomoserine

- A 10 μ L aliquot of the above sample is analyzed by ion chromatography using a Dionex DX-500 apparatus fitted with a 2 mm AG-11 precolumn and a 2 mm AS-11 column, an ASRS Ultra suppressor, and a 10 μ L injection loop. A gradient is then applied: 0 – 7 min 0.5 mM KOH; 7 min injection; 7 – 9.5 min 0.5 mM KOH; 9.5 – 13 min 0.5 – 5 mM KOH; 13 – 25 min 5 – 38.3 mM KOH.

Measurement of the methionine synthase activity by detection of the quantity of methionine synthesized in the presence of methylmercaptan

- The analysis is carried out by GC-MS, which requires the silylation of samples before injection. For this purpose each sample receives an internal standard (serine 13C) to allow the quality of the silylation to be validated. The samples are then lyophilized overnight.

The derivatization is carried out using the following protocol:

Using a 1 ml automatic pipette 400 μ l of hydroxylamine solution (0.250 g \pm 0.002 g dissolved in 10 ml of pyridine) is added, making sure the tubes were tightly closed. The mixture is vortexed twice for 10 seconds, centrifuged to concentrate it at the bottom of the tube (max. 1 minute at 5000 g) and left to react for 1½ hours at 30°C. The tubes are opened and 1000 μ l of BSTFA solution is added using a 1 ml automatic pipette, topping up with 100 μ l of pyridine (200 μ l automatic pipette). The tubes are closed, vortexed for 10 seconds and left to incubate respectively for 60 minutes at 60°C for TBDMS derivatives and 30 minutes at 70°C for BSTFA. If necessary the samples are filtered on a disposable filter with a 0.22 μ m PTFE membrane or centrifuged at 5000 g for 5 minutes. They are transferred to 1.5 ml flasks, sealed and injected into the GC.

The analyses are carried out with an Agilent Technologies GC6890/MSD5973 apparatus fitted with a non-polar column (HP-5MS, Bios Analytique). The carrier gas is helium with a constant flow rate of 1 ml.min⁻¹. The injection of 1 μ l of sample is in splitless mode with a purge flow rate of 50 ml.min⁻¹ for 0.85 min. The temperature profile is: initial temperature 90°C maintained for 2 minutes and then increased to 320°C with a gradient of 10°C.min⁻¹. This temperature is maintained for 6 minutes. Detection is by mass spectrometry with ionization by electron impact in scanning mode in the range m/z = 40 to 550 amu. The solvent passage time is set at 3.10 minutes.

Under these conditions, a 'methionine synthase' activity can be assayed in the samples incubated with methanethiol, by the quantification first of succinate by ion chromatography and second of methionine by GC-MS.

The results are given in Table 4 below.

| Strain | Protein concentration | Specific activity (mUI/mg protein) | |
|--------|-----------------------|------------------------------------|------------------|
| | | Succinate assay | Methionine assay |
| MBM01 | 3.43 | 0.30 | 0.23 |
| K183 | 3.62 | 1.40 | 1.72 |

Table 4: Methionine synthase activity in the presence of methylmercaptan of extracts from strains MBM01 and K183

It is thus evident that the methionine synthase activity in the presence of methylmercaptan is strengthened in the evolved strain relative to the wild strain, confirming that the mutated cystathionine γ -synthase (E325A) has a modified methionine synthase activity.

e) Isolation of the evolved gene and kinetic characterization of the enzyme METB* possessing an evolved methionine-synthase activity

- 10 To determine the kinetic parameters of the methionine-synthase and cystathionine- γ -synthase activities exerted by METB and METB*, the genes *metB* and *metB** are cloned in an overexpression vector pTopo (Invitrogene) using the following strategy:
 - Amplification of the gene *metB* or *metB** with the oligonucleotides *metJ* / *metLR* and heat-resistant polymerase *Pwo*.
- 15 - Ligation of the PCR product to the plasmid pTOPO 4-PCR blunt, and introduction of the plasmid thus formed, pTopo.*metB* or pTopo.*metB** into DH5 α and selection of Ap^r clones.
 - Verification by enzymatic digestion of the configuration of the plasmid pTopo.*metB* ou pTopo.*metB** after extraction.
- 20 - Introduction of the verified plasmid pTopo.*metB* into the strain MG1655(Δ *metBJ*, Δ *metE*). Verification by enzymatic digestion, as previously described, of the introduced plasmid. Verification by PCR of the strain MG1655(Δ *metBJ*, Δ *metE*) with the oligonucleotides *metJR* / *metLR* for the deletion *metJ*, and *metER* / *metEF* for the deletion *metE*: strain MINS33.

Cultures are then carried out on rich medium and protein extracts are prepared. The methionine-synthase and cystathionine- γ -synthase enzyme activities are then determined using sodium methylmercaptide and cysteine as reaction co-substrates, respectively.

- 5 The kinetic characteristics of the methionine synthase activity are given in Table 5. The kinetic characteristics of the cystathionine- γ -synthase activity are given in Table 6.

| | K_m | V_{max} |
|------------|--------|---------------------|
| pTOPOmetB | 277 mM | 13.9 mUI/mg protein |
| PTOPOmetB* | 6 mM | 5.6 mUI/mg protein |

Table 5. Apparent kinetic characteristics of the methionine-synthase activity of enzymes METB and METB*.

- 10 The effect of the mutation A325E is to reduce the K_m of the enzyme 45-fold for methylmercaptan, whereas V_{max} is only halved.

| | K_m | V_{max} |
|------------|--------|----------------------|
| pTOPOmetB | 7.5 mM | 39840 mUI/mg protein |
| PTOPOmetB* | 0.6 mM | 2889 mUI/mg protein |

Table 6. Apparent kinetic characteristics of the cystathionine- γ -synthase activity of enzymes METB and METB*.

- 15 The effect of the mutation A325E reduces the cystathionine- γ -synthase activity 13-fold and the K_m of the enzyme for cysteine.

Example F.I.2.: Evolution of a homocysteine synthase activity from a cystathionine- γ -synthase

• Construction of strains MG1655 ($\Delta metC::Cm$) and MG1655 ($\Delta metC$)

- 20 To inactivate the gene *metC* the homologous recombination strategy described by Datsenko & Wanner (2000) is used. This strategy permits the insertion of a chloramphenicol resistance cassette, while deleting most of the gene concerned. For this purpose 2 oligonucleotides were used:

For *metC*:

- DmetCR with 100 bases (SEQ ID NO 13):

ccggcggtccagatcggcaatcagatcgtcgacatcttccagaccaatatgcaggcgaatcaaggtcccgttaa
aatcgatCATATGAATATCCTCCTTAG

with

- a region (lower case) homologous to the sequence (3151419 to 3151359) of
5 the gene *metC* (sequence 3150251 to 3151438, reference sequence on the website
<http://genolist.pasteur.fr/Colibri/>),

- a region (upper case) for the amplification of the chloramphenicol resistance
cassette (reference sequence in Datsenko, K.A. & Wanner, B.L., 2000, PNAS, 97:
6640-6645),

- 10 - DmetCF with 100 bases (SEQ ID NO 14):

cggacaaaaagcttgataactcaactggtgaatgcaggacgcagcaaaaaatacactctcggcgcggttaaata
gcgtgattTGTAGGCTGGAGCTGCTTCG

with

- a region (lower case) homologous to the sequence (3150255 to 3150334) of
15 the gene *metC*
- a region (upper case) for the amplification of the chloramphenicol resistance
cassette.

The oligonucleotides DmetCR and DmetCF are used to amplify the
chloramphenicol resistance cassette from the plasmid pKD3. The PCR product
20 obtained is then introduced by electroporation into the strain MG1655 (pKD46) in
which the Red recombinase enzyme expressed permits the homologous
recombination. The chloramphenicol resistant transformants are then selected and the
insertion of the resistance cassette is verified by a PCR analysis with the
oligonucleotides metCR and metCF defined previously. The strain retained is
25 designated MG1655 ($\Delta metC::Cm$).

MetCR (SEQ ID NO 11): cgtccgggacgccttgatcccggacgcaac (homologous to
the sequence from 3151522 to 3151493).

MetCF (SEQ ID NO 12): gcgtttacgcagtaaaaaagtcaccagcacgc (homologous to
the sequence from 3150118 to 3150149).

- 30 The chloramphenicol resistance cassette can then be eliminated. The plasmid
pCP20 carrying recombinase FLP acting at the FRT sites of the chloramphenicol
resistance cassette is then introduced into the recombinant strains by electroporation.

After a series of cultures at 42°C, the loss of the chloramphenicol resistance cassette is verified by a PCR analysis with the same oligonucleotides as those used previously. The strain retained is designated MG1655 ($\Delta metC$).

The construction of the strain $\Delta(metC)$ is described in Example F.I.2. In a particular embodiment of the invention the strain *E. coli* $\Delta(metC)$ is cultured in flasks (see Example F.I.1) containing a minimal medium with glucose as sole carbon source. The medium contained neither methylmercaptan nor H₂S. Reseeding is carried out and growth rates are determined for each reseeded. A very marked improvement in the growth rate of the strain $\Delta(metC)$ is observed on the minimal medium, suggesting that the homocysteine synthase activity of the cystathionine γ -synthase is strongly improved in the presence of endogenous H₂S (see Figure 10).

| Reseeding cycle number | measured μ (h ⁻¹) |
|------------------------|-----------------------------------|
| 1 | 0.05 |
| 3 | 0.37 |
| 5 | 0.39 |
| 10 | 0.44 |
| 12 | 0.44 |

Example F.I.3.: Evolution of a methionine synthase activity from an acetylhomoserine sulfhydrylase activity.

Construction of the strain MG1655 ($\Delta metB$ - $\Delta metJ$)

To delete the genes *metB* and *metJ*, and conserve the promoter of the operon *metBL*, a chloramphenicol resistance cassette is inserted, while at the same time deleting most of the genes concerned and maintaining the promoter of *metBL*. For this purpose we use 2 oligonucleotides.

For *metBJ*:

20 - MetJR with 30 bases (SEQ ID NO 9):

ggtacagaaaccagcaggctgaggatcagc

homologous to the sequence (4125431 to 4125460) downstream of the gene *metJ* (sequence 4125975 to 4125581, reference sequence on the website <http://genolist.pasteur.fr/Colibri/>).

25 - DmetJBF with 100 bases (SEQ ID NO 10):

tatgcagctgacgaccttcgccctgctgcgcaatcacactcattttacccttggttcagcccgggaagcca
tttccaggcaccagagtaaacatt

with

- a part (upper case) homologous to the sequence (4126217 to 4126197)
5 between the genes *metJ* and *metB* (sequence 4126252 to 4127412) containing the promoter region of the operon *metBLF*,
- a part (lower case) homologous to the sequence (4127460 to 4127380) corresponding to the beginning of the gene *metL* (sequence 4127415 to 4129847) and the end of the gene *metB*.

10 These two oligonucleotides were used to amplify the region concerned on the chromosomal DNA of MG1655 $\Delta(\text{metJ}::\text{Cm})$; see Figure 11.

The PCR product obtained is then introduced by electroporation into the strain MG1655 (pKD46) in which the Red recombinase enzyme expressed permits the homologous recombination. The chloramphenicol resistant transformants are then
15 selected and the deletion of the gene *metB* by homologous recombination is verified by PCR analysis with the oligonucleotides MetJR and MetLR, defined previously.

The desired strain is strain MG1655 ($\Delta\text{metB}-\Delta\text{metJ}::\text{Cm}$) in which the genes *metJ* and *metB* are eliminated and the promoter of the operon *metBLF* repositioned before *metL* (see Figure 12).

20 The chloramphenicol resistance cassette can then be eliminated. The plasmid pCP20 carrying FLP recombinase acting at the FRT sites of the chloramphenicol resistance cassette is then introduced into the recombinant strains by electroporation. After a series of cultures at 42°C, the loss of the chloramphenicol resistance cassette is verified by PCR analysis with the same oligonucleotides as used previously
25 (MetLR and MetJR).

Construction of the strains MG1655 $\Delta(\text{metC}::\text{Cm}, \text{metJB})$ and MG1655 $\Delta(\text{metC}, \text{metJB})$

To delete the gene *metC* (sequence 3150251 to 3151438, the reference sequence on the website <http://genolist.pasteur.fr/Colibri/>) of the strain MG1655
30 ($\Delta\text{metB}-\Delta\text{metJ}$), the method of phage P1 transduction is used. The protocol followed is implemented in 2 steps with the preparation of the phage lysate on the strain MG1655 $\Delta(\text{metC}::\text{Cm})$ and then transduction into strain MG1655 $\Delta(\text{metB}-\Delta\text{metJ})$.

The construction of the strain $\Delta(\text{metC}::\text{Cm})$ is described in Example F.I.2.

Preparation of phage lysate P1:

- Seeding with 100 μl of an overnight culture of the strain MG1655 ($\Delta\text{metC}::\text{Cm}$) of 10 ml of LB + Cm 30 $\mu\text{g}/\text{ml}$ + glucose 0.2% + CaCl_2 5 mM.
- 5 - Incubation for 30 min at 37°C with shaking.
- Addition of 100 μl of phage lysate P1 prepared on the wild strain MG1655 (about 1.10^9 phage/ml)
- Shaking at 37°C for 3 hours until all the cells were lysed.
- Addition of 200 μl of chloroform and vortexing.
- 10 - Centrifuging for 10 min at 4500 g to eliminate cell debris.
- Transfer of supernatant to a sterile tube and addition of 200 μl of chloroform.
- Storage of lysate at 4°C.

Transduction

- Centrifuging for 10 min at 1500 g of 5 ml of an overnight culture of the strain
- 15 MG1655 ($\Delta\text{metB}-\Delta\text{metJ}$) in LB medium.
- Suspension of the cell pellet in 2.5 ml of 10 mM MgSO_4 , 5 mM CaCl_2
- Control tubes: 100 μl cells
100 μl phages P1 of strain MG1655 ($\Delta\text{metC}::\text{Cm}$)
- Test tube: 100 μl of cells + 100 μl of phages P1 of the strain MG1655
- 20 ($\Delta\text{metC}::\text{Cm}$)
- Incubation for 30 min at 30°C without shaking.
- Addition of 100 μl of 1 M sodium citrate in each tube and vortexing.
- Addition of 1 ml of LB
- Incubation for 1 hour at 37°C with shaking
- 25 - Spreading on dishes LB + Cm 30 $\mu\text{g}/\text{ml}$ after centrifuging of tubes for 3 min at 7000 rpm.
- Incubation at 37°C overnight.

Verification of the strain

- The chloramphenicol transformants are then selected and the insertion of the
- 30 region containing ($\text{metC}::\text{Cm}$) is verified by a PCR analysis with the oligonucleotides MetCR and MetCF, and MetJR and MetLR to verify also the strain with genes *metB* and *metJ*. The strain retained is designated MG1655 $\Delta(\text{metC}::\text{Cm}, \text{metJB})$.

MetCR (SEQ ID NO 11): cgctccgggacgccttgatcccgggacgcaac (homologous to sequence 3151522 à 3151493)

MetCF (SEQ ID NO 12): gcgtttacgcagtaaaaaagtcaccagcacgc (homologous to the sequence from 3150118 to 3150149).

5 As previously, the chloramphenicol resistance cassette can then be eliminated. The plasmid pCP20 carrying FLP recombinase acting at the FRT sites of the chloramphenicol resistance cassette is then introduced into the recombinant sites by electroporation. After a series of cultures at 42°C, the loss of the chloramphenicol resistance cassette is verified by a PCR analysis with the same oligonucleotides as
10 used previously (MetCR and MetCF, and MetJR and Met LR). The strain retained is designated MG1655 $\Delta(\text{metC}, \text{metJB})$.

Introduction of the plasmid pTrc99A-metY and evolution of the strain

In a particular embodiment, a plasmid is constructed that permits the expression of the gene *metY* of *C. glutamicum*. This gene is amplified by PCR from
15 chromosomal ADN of *C. glutamicum* and introduced into a plasmid pTrc99A. It is possible to amplify by PCR the gene *metY* and if necessary its natural promoter. In a preferred embodiment, the gene *metY* is cloned under the control of a promoter that permits an expression in *E. coli*. The vector used is a pTrc99A (Pharmacia), but a vector selected from among pUC, pBluescript, pCR-Script, pTopo, etc. could also be
20 used.

The strain *Escherichia coli* $\Delta(\text{metC}, \text{metJB})$ obtained previously is transformed with the plasmid pTrc-*metY* of *C. glutamicum*. The transformation of the strain is carried out by electroporation.

The strain obtained is then inoculated in a conical flask ($\text{OD}_{600\text{nm}} \sim 0.4 - 0.5$)
25 containing 10% of its volume in a minimal medium with glucose for sole carbon source. The low succinylhomoserine sulfhydrylase activity initially carried by the enzyme MetY limits the growth ($\mu \sim 0.06 \text{ h}^{-1}$) of the bacterial population on the minimal medium (MML8) owing to limitation of synthesized methionine. Reseeding is carried out when the $\text{OD}_{600\text{nm}}$ in the flask reached about 2. The selection is thus
30 conducted for 22 culture cycles. A marked improvement in the growth rate is observed during this phase of evolution-selection (Figure 13). In view of prior experience, it is likely that the improvement in growth observed corresponds to an

evolution of the gene *metY* such that the O-acetyl-homoserine sulphydrylase activity is changed into an O-succinyl-homoserine sulphydrylase activity allowing the production of homocysteine from O-succinylhomoserine and H_2S ; these two substrates being produced by the bacterium.

- 5 To optimize the process of evolution of *metY*, a similar approach is possible using other mutants of *Escherichia coli*, in particular the mutant $\Delta(\textit{metC}, \textit{metB})$.

Example F.I.4.: Fed-batch culture process for the production and purification of methionine

Pre-culture

- 10 The pre-culture is carried out overnight in a 500 ml flask containing 50 ml of minimal medium, type M9 modified, supplemented with 2.5 g/l of glucose. The cells are recovered by centrifuging and taken up in 5 ml of minimal medium, type M9 modified.

Culture in a fermenter

- 15 The culture is carried out in a fermenter with a useable volume of 300 ml of the Fedbatch-pro DASGIP type.

The fermenter is filled with 145 ml of minimal medium, type M9 modified, and inoculated with 5 ml of pre-culture, i.e., an inoculation OD_{600nm} between 0.5 and 1.2.

- 20 The temperature of the culture is maintained between 30 and 37°C and the pH is continuously adjusted to a value between 6.5 and 8. It is partially regulated by adding a solution of CH_3SNa . A solution of 2N sodium hydroxide can if necessary be used to complete the regulation. Shaking is maintained at between 200 and 400 rpm during the batch phase and is increased to 1000 rpm at the end of the fed-batch process.
- 25 The dissolved O_2 content is maintained between 30% and 40% saturation using a gas controller. As soon as the OD_{600nm} attains a value between 2.5 and 3 the fed-batch process is started by adding the fed medium at an initial flow rate of between 0.3 and 0.5 ml/h with a gradual increase to flow rates between 2.5 and 3.5 ml/h. Thereafter the flow rate is maintained constant for between 24 h and 32 h. The
- 30 fed medium is made up on the basis of a modified M9 medium complemented by a glucose concentration between 300 and 500 g/l of glucose. At the same time the medium is supplemented with a solution of CH_3SNa (solution between 1 and 5 M) to

allow bacterial growth while at the same time regulating the pH. As soon as the cell concentration reaches a value between 20 and 50 g/l the fed medium is replaced by a minimal medium of type M9 with limited phosphorus. The solution of methylmercaptan is replaced by a direct injection of CH₃SH in gaseous form into the fermenter. The gas flow rate is adapted to the flow rate of the fed solution in molar ratios to the carbon substrate ranging from 1 to 3. As soon as the cell concentration is between 50 and 80 g/l the fermentation is stopped. The pH of the fermentation must liquor is adjusted to between 7.5 and 9 with a solution of NaOH, and it is heated to between 60 and 80°C. The liquor is then filtered on UF modules. The temperature of the liquor is maintained at between 60 and 80°C, and the liquor is then concentrated before running it through charcoal to de-color it (in a column or batchwise). The de-colored liquor is filtered again to remove last particles before acidification with concentrated HCl to a pH below 2.28 (pK₁ of methionine). The crystals of methionine hydrochloride thus formed are recovered by filtration and the HCl eliminated by evaporation, yielding purified L-methionine.

Registration of biological material

The strain K183 was registered on April 2 2003 at the Collection Nationale de Cultures de Microorganismes (CNCM), 25 rue du Docteur-Roux, 75724 Paris Cedex 15, France, in compliance with the provisions of the Treaty of Budapest, under the serial number I-3005.

F. II. Evolution of the cysteine biosynthesis pathway

One application (Example F.II.1.) of the invention to metabolic engineering of the biosynthesis pathway of cysteine comprises the following steps:

- a) Deletion of the genes *cysK*, *cysM* in the initial strain of *E. coli*; the modified strain obtained is thus auxotrophic to cysteine. The initial strain is able to grow on a minimal medium (MM) containing no methionine, S-adenosylmethionine, homocysteine, cystathionine, or cysteine, whereas the modified strain has lost that ability.
- a1) Introduction of the gene *metY*, a heterologous gene from *C. glutamicum*. This gene is to evolve from an acetylhomoserine sulphydrylase activity into a cysteine-synthase activity.

- b) Culture of the modified strain *E. coli* [*metY* Δ (*cysK*, *cysM*)] on the same minimal medium (MM) with no co-substrate, to cause the evolution of MetY into a cysteine-synthase activity to compensate for the initially deleted enzyme activities (CysK, CysM).
- 5 c) Selection of an evolved strain with a new cysteine-synthase activity in the presence of endogenous H₂S; verification of the new synthesis pathway.

Example F.II.1: Evolution of an acetylhomoserine sulphydrylase activity into a cysteine synthase activity

a) Construction of the strain *E. coli* Δ (*cysK*, *cysM*)

- 10 The inactivation of the genes *cysK* and *cysM* is carried out by inserting an antibiotic resistance cassette (chloramphenicol and kanamycin respectively) while at the same time deleting most of the genes concerned. The method used is described by Datsenko, K.A.; Wanner, B.L. (2000), One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97: 6640-6645. For each construction a pair of oligonucleotides is synthesized:

For *cysK*:

DcysKR with 100 bases (SEQ ID NO 15):

TgttgcaattctttctcagtggaagatcggaacaatgcggtgcttaataacgctcacccgatgatggtagaataacC
ATATGAATATCCTCCTTAG

20 with

- a region (lower case) homologous to the sequence (2531396 to 2531317) of the gene *cysK* (reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),
- a region (upper case) for the amplification of the chloramphenicol resistance cassette (reference sequence in Datsenko, K.A. & Wanner, B.L., 25 2000, PNAS, 97: 6640-6645).

DcysKF de 100 bases (SEQ ID NO 16):

agtaagatttttgaagataactcgctgactatcggtcacacgccgctggttcgcctgaatcgcatcggtaacggacgcatT
GTAGGCTGGAGCTGCTTCG

30 with:

- a region (lower case) homologous to the sequence (2530432 à 2530511) of the gene *cysK*,

- a region (upper case) for the amplification of the chloramphenicol resistance cassette.

Pour *cysM*:

DcysMR with 100 bases (SEQ ID NO 17):

5 cccgccccctggctaaaatgctcttccccaaacaccccggtagaaaggtagcgatcgccacgatcgagatgatcgcca
cCATATGAATATCCTCCTTAG

with:

- a region (lower case) homologous to the sequence (2536699 to 2536778) of the gene *cysM* (reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),
- a region (upper case) for the amplification of the kanamycin resistance cassette.

DcysMF with 100 bases (SEQ ID NO 18):

Agtacattagaacaacaataggcaatacgcctctggtgaagttgcagcgaatggggccggataacggcagtggaagtgt
15 gTG TAGGCTGGAGCTGCTTCG

with:

- a region (lower case) homologous to the sequence (2537600 to 2537521) of the gene *cysM*,
- a region (upper case) for the amplification of the kanamycin resistance cassette.

The oligonucleotides DcysKR and DcysKF, and DcysMR and DcysMF are used respectively to amplify the chloramphenicol and kanamycin resistance cassettes from plasmids pKD3 and pKD4. The PCR product obtained is then introduced by electroporation in the strain MG1655 (pKD46) in which the enzyme Red recombina-
25 se expressed permits the homologous recombination. The transformants resistant to each of the antibiotics are then selected and the insertion of the resistance cassette is verified by a PCR analysis with the oligonucleotides *cysKR* and *cysKF*, and *cysMR* and *cysMF*.

cyKR (SEQ ID NO 19): ttttaacagacgcgacgcacgaagagcgc (homologous to the
30 sequence from 2531698 to 2531669)

cysKF (SEQ ID NO 20): ggcgcgacggcgatgtgggtcgattgctat (homologous to the sequence from 2530188 to 2530217)

cysMR (SEQ ID NO 21): ggggtgacggtcaggactaccaatacttc (homologous to the sequence from 2536430 to 2536459)

cysMF (SEQ ID NO 22): gcgcgcacgctggccgctgggctacacac (homologous to the sequence from 2538071 to 2538042).

5 The chloramphenicol and kanamycin resistance cassettes can then be eliminated. For this purpose the plasmid pCP20, carrying FLP recombinase acting at the FRT sites of the chloramphenicol or kanamycin resistance cassettes, is introduced into the recombinant strains by electroporation. After a series of cultures at 42°C, the loss of the antibiotic resistance cassette is verified by a PCR analysis with the same
10 oligonucleotides as used previously.

a1) Introduction of the gene *metY* in the preceding strain

The plasmid pTopometY is constructed by insertion of the gene *metY* in the vector Zero Blunt TOPO PCR cloning kit (PCR4 TOPO vector, Invitrogen). For this purpose the gene *metY* is amplified by PCR with the polymerase Pwo from the
15 chromosomal DNA of the strain *Corynebacterium glutamicum* ATCC13032 using the following oligonucleotides:

MetYR (SEQ ID NO 23):

ttagagctgttgacaattaatcatccggctcgataatgtgtggaataaaaaactctta**aggac**ctccaaatgcc

Promoter of type TRC (pTRC-O) in bold roman, RBS of the gene *metY* in
20 bold roman underlined, initiation codon of the gene *metY* in bold italics.

MetYF (SEQ ID NO 24):

gctctgtctagtctagttgcattctcag

Sequence chosen downstream of the transcription terminator metY.

The PCR product obtained is then directly cloned in the vector Topo to give
25 the plasmid pTopometY. The vector Topo carries a replication origin for *E. coli*, an ampicillin resistance gene and a kanamycin resistance gene.

The plasmid pTopometY is then introduced into the strain *E. coli* DH5α for verification of the construction. The sequencing of the gene *metY* of the plasmid pTopometY with the universal oligonucleotides M13 reverse and M13 forward is
30 then carried out to confirm the construction.

The plasmid is introduced into the strain *E. coli* Δ(*cysK*, *cysM*) by electroporation.

c) Culture of the modified strain to cause the gene *metY* coding for acetyl-homoserine sulfhydrylase activity to evolve toward a cysteine synthase activity

The controlled selection of the preceding strain containing the gene *metY* can be carried out in bottles or conical flasks. The implementation of this method permits the selection of a strain of *Escherichia coli* in which the enzyme acetyl-homoserine sulfhydrylase has evolved into a 'cysteine synthase' activity. The controlled selection is performed in conical flasks containing 50 ml of inorganic medium (Schaefer et al., 1999, *Anal. Biochem.* 270: 88-96) in the presence of 33 mM glucose, chloramphenicol at a final concentration of 25 mg/l and kanamycin at a concentration of 25 mg/l.

The culture media are seeded with the strain *E. coli* K12 [$\Delta((cysK, cysM) pTopometY)$] at a defined OD_{600nm} value. Seeding is carried out with a population of bacteria sufficiently large for some bacteria potentially to possess relevant mutations in the gene *metY* enabling them to assimilate O-acetyl serine. This population is obtained by growing the strain auxotrophic for cysteine on a cysteine-supplemented minimal medium. A control culture is seeded with the strain *E. coli* K12 ($\Delta cysK, cysM$).

The cultures are carried out with shaking at 37°C, for 6 days, after which time the OD_{600nm} is measured. The control culture displays practically no change in OD, while some other cultures exhibit significant evolution of their OD. It is therefore probable that the 'evolved cysteine synthase activity' has appeared in the populations contained in those conical flasks. The mutation or mutations probably occur in the gene *metY* because this gene was the only difference between these strains and the control strain.

The bacterial population of these positive cultures can then be used to further improve the cysteine synthase activity by repeating the flask culture procedure as described.

c) Selection of clones

The evolved population is then spread on a gelosed minimal medium containing glucose as sole carbon source. The inoculated dishes are placed in aerobic conditions in an incubator at 37°C. After 36 hours, the clones appear on the dishes;

they correspond to bacteria able to produce cysteine from glucose as sole carbon source. Three clones are isolated

• **Control of the synthesis pathway**

The population of evolved *E. coli* K12 [Δ (*cysK*, *cysM*) pTopometY] is
 5 cultured in a minimal medium (Schaefer *et al.*, 1999, *Anal. Biochem.* **270**: 88-96) containing 2,5 g.l⁻¹ of glucose uniformly labeled with carbon 13. After culture, the cells were recovered, washed and hydrolysed with 6N HCl for 24 hours at 107°C. A 2D NMR analysis was then performed (HSQC). This analysis allows the fate of the glucose carbon 13 to be determined, thereby confirming that the synthesis of cysteine
 10 takes place via serine and acetyl-serine, indicating that the enzyme coded for by the gene *metY* has evolved into a cysteine synthase.

F. III. Evolution of NADPH-dependent enzymes

One application (Example F.III.1.) of the invention to metabolic engineering of NADPH-dependent bioconversion pathways comprises the following steps:

- 15 a1) Inactivation of the genes *udhA* and *pgi* in the initial strain *E. coli*;
- a2) Inactivation of the genes *pfkA*, *pfkB* and *udhA* in the initial strain *E. coli*;
- The resulting modified strains are thus optimized for their ability to reduce NADP. The initial strain is able to grow on a minimal medium (MM), while the ability of the modified strains to grow on that medium is strongly impaired.
- 20 b) Introduction of a plasmid harboring the gene *yueD* coding for a benzyl reductase of *Bacillus cereus*.
- c) Culture of the preceding modified strain on the minimal medium (MM) to which is added p-nitrobenzaldehyde (co-substrate) to cause the evolution of the benzyl reductase activity for that poorly metabolized substrate .
- 25 d) Characterization of the evolved enzyme YueD

Example F.III.1: Construction of the strains *E. coli* Δ (*udhA*, *pgi*) and *E. coli* Δ (*pfkA*, *pfkB*, *udhA*) and modification of the kinetic characteristics of the benzyl reductase of *Bacillus cereus*

a1) Construction of the modified strain *E. coli* Δ (*udhA*, *pgi*)

- 30 The inactivation of the gene *udhA*, is carried out by inserting an antibiotic resistance cassette conferring resistance to kanamycin while at the same time

deleting most of the gene concerned. The method used is described by Datsenko, K.A.; Wanner, B.L. (2000), One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**: 6640-6645.

5 For this purpose two oligonucleotides are synthesized:

- DudhAR with 100 bases (SEQ ID NO 25):

cccagaatctcttttgtttcccgatggaacaaaattttcagcgtgccacgttcacgacgattgtgcgcgtg
ccagTGTAGGCTGGAGCTGCTTCG

with

- 10 - a region (lower case) homologous to the sequence (4157144 to 4157223) of the gene *udhA* (sequence 4158303 to 4156969, reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),
- a region (upper case) for the amplification of the kanamycin resistance cassette (reference sequence in Datsenko, K.A. & Wanner, B.L., 2000, PNAS, 97: 6640-6645).

15 DudhAF with 100 bases (SEQ ID NO 26):

ggtgcgcgcgtcgagttatcgagcgttatcaaatgttgcgcggttgacccactggggcaccatcccgtcgaaag
cCATATGAATATCCTCCTTAG

with:

- 20 - a region (lower case) homologous to the sequence (4158285 to 4158206) of the gene *udhA*,
- a region (upper case) for the amplification of the kanamycin resistance cassette.

The oligonucleotides DudhAR and DudhAF are used to amplify the
25 kanamycin resistance cassette from the plasmid pKD4. The PCR product obtained is then introduced by electroporation into the strain MG1655 (pKD46) in which the Red recombinase enzyme expressed permits the homologous recombination. The kanamycin resistant transformants are then selected and the insertion of the resistance cassette is verified by a PCR analysis with the oligonucleotides UdhAR
30 and UdhAF

UdhAR (SEQ ID NO 27) : gcgggatcactttactgccagcgtggctg (homologous to the sequence 4156772 to 4156801)

UdhAF (SEQ ID NO 28) : ggccgctcaggatatagccagataaatgac (homologous to the sequence 4158475 to 4158446)

The inactivation of the gene *pgi*, is carried out by inserting an antibiotic resistance cassette conferring resistance to chloramphenicol while at the same time
 5 deleting most of the gene concerned. The method used is described by Datsenko, K.A.; Wanner, B.L. (2000), One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97: 6640-6645.

For this purpose two oligonucleotides are synthesized:

10 - DpgiR with 100 bases (SEQ ID NO 29):

gcgccacgctttatagcggtaatcagaccattggtcgagctatcgtggctgctgatttctttatcatctttcagctc
 tgCATATGAATATCCTCCTTAG

with

- a region (lower case) homologous to the sequence (4232980 to 4232901)
 15 of the gene *pgi* (sequence 4231337 to 4232986, reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),
- a region (upper case) for the amplification of the chloramphenicol resistance cassette (reference sequence in Datsenko, K.A. & Wanner, B.L., 2000, PNAS, 97: 6640-6645).

20 - DpgiF with 100 bases (SEQ ID NO 30):

ccaacgcagaccgctgcctggcaggcactacagaaacacttcgatgaaatgaaagacgttacgatcgccgatcttttgc
 TGTAGGCTGGAGCTGCTTCG

with:

- a region (lower case) homologous to the sequence (4231352 to 4231432)
 25 of the gene *pgi*,
- a region (upper case) for the amplification of the chloramphenicol resistance cassette.

The oligonucleotides DpgiR and DpgiF are used to amplify the chloramphenicol resistance cassette from the plasmid pKD3. The PCR product
 30 obtained is then introduced by electroporation into the strain MG1655 Δ *udhA* (pKD46) in which the Red recombinase enzyme expressed permits the homologous recombination. The chloramphenicol resistant transformants are then selected and the

insertion of the resistance cassette is verified by a PCR analysis with the oligonucleotides PgiR and PgiF

PgiR (SEQ ID NO 31) : cggatatgattccgtaaattacagacaag (homologous to the sequence 4233220 to 4233191)

5 PgiF (SEQ ID NO 32) : gcggggcggttgtaacgatggggcatgc (homologous to the sequence 4231138 to 4231167)

The two antibiotic resistance cassettes can then be eliminated. The plasmid pCP20 carrying recombinase FLP acting at the FRT sites of both the kanamycin and chloramphenicol resistance cassette is then introduced into the recombinant strains
10 by electroporation. After a series of cultures at 42°C, the loss of the two resistance cassettes is verified by a PCR analysis with the same oligonucleotides as those used previously.

a2) Construction of the modified strain *E. coli* $\Delta(pfkA, pfkB, udhA)$

The inactivation of the gene *pfkA*, is carried out by inserting an antibiotic
15 resistance cassette conferring resistance to chloramphenicol while at the same time deleting most of the gene concerned. The method used is described by Datsenko, K.A.; Wanner, B.L. (2000), One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97: 6640-6645.

20 For this purpose two oligonucleotides are synthesized:

- DpfkAR with 100 bases (SEQ ID NO 33):

ttcgcgcagtccagccagtcaccttgaacggacgcttcattgtttcgaagcgtcgatgatgtcgtggtgaacc
agctgCATATGAATATCCTCCTTAG

with

- 25 - a region (lower case) homologous to the sequence (4106081 to 4106002) of the gene *pfkA* (sequence 4105132 to 4106094, reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),
- a region (upper case) for the amplification of the chloramphenicol resistance cassette (reference sequence in Datsenko, K.A. & Wanner, B.L.,
30 2000, PNAS, 97: 6640-6645).
- DpfkAF de 100 bases (SEQ ID NO 34):

ggtgtgttgacaagcggcgggtgatgcgccaggcatgaacgccgcaattcgcggggtgttcgttctgcgctgacagaa
ggTGTAGGCTGGAGCTGCTTCG

with:

- a region (lower case) homologous to the sequence (4105147 to 4105227)
5 of the gene *pfkA*,
- a region (upper case) for the amplification of the chloramphenicol
resistance cassette.

The oligonucleotides DpfkAR and DpfkAF are used to amplify the
chloramphenicol resistance cassette from the plasmid pKD3. The PCR product
10 obtained is then introduced by electroporation into the strain MG1655 (pKD46) in
which the Red recombinase enzyme expressed permits the homologous
recombination. The chloramphenicol resistant transformants are then selected and the
insertion of the resistance cassette is verified by a PCR analysis with the
oligonucleotides PfkAR and PfkAF

15 PfkAR (SEQ ID NO 35) : ccctacgccccacttgttcacgccc (homologous to the
sequence 4106434 to 4106408)

PfkAF (SEQ ID NO 36) : cgcacgcggcagtcagggccgaccgc (homologous to the
sequence 4104751 to 4104777)

The inactivation of the *udhA* gene was described in example F.III.1 a1) and
20 can be carried out in the same way.

The two antibiotic resistance cassette can then be eliminated. The plasmid
pCP20 carrying recombinase FLP acting at the FRT sites of both the kanamycin and
chloramphenicol resistance cassette is then introduced into the recombinant strains
by electroporation. After a series of cultures at 42°C, the loss of the two resistance
25 cassettes is verified by a PCR analysis with the same oligonucleotides as those used
previously.

The inactivation of the gene *pfkB*, is carried out by inserting an antibiotic
resistance cassette conferring resistance to chloramphenicol while at the same time
deleting most of the gene concerned. The method used is described by Datsenko,
30 K.A.; Wanner, B.L. (2000), One-step inactivation of chromosomal genes in
Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97: 6640-
6645.

For this purpose two oligonucleotides are synthesized:

- DpfbBR with 100 bases (SEQ ID NO 37):

gcgggaaaggtaagcgtaaatttttgcgtatcgatgggagcacagacgtgtccctgattgagtggtgc
actccCATATGAATATCCTCCTTAG

5 with

- a region (lower case) homologous to the sequence (1805320 to 1805241) of the gene *pfbB* (sequence 1804394 to 1805323, reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),
- a region (upper case) for the amplification of the chloramphenicol resistance cassette (reference sequence in Datsenko, K.A. & Wanner, B.L., 10 2000, PNAS, 97: 6640-6645).

- DpfbBF with 100 bases (SEQ ID NO 38):

gcgccctctctcgatagcgcaacaattaccccgcaaatattatcccgaaaggaaaactgcgctgtaccgcaccggtgttcgT
GTAGGCTGGAGCTGCTTCG

15 with:

- a region (lower case) homologous to the sequence (1804421 to 1804499) of the gene *pfbB*,
- a region (upper case) for the amplification of the chloramphenicol resistance cassette.

20 The oligonucleotides DpfbBR and DpfbBF are used to amplify the chloramphenicol resistance cassette from the plasmid pKD3. The PCR product obtained is then introduced by electroporation into the strain MG1655 $\Delta(pfbA, udhA)$ (pKD46) in which the Red recombinase enzyme expressed permits the homologous recombination. The chloramphenicol resistant transformants are then selected and the
25 insertion of the resistance cassette is verified by a PCR analysis with the oligonucleotides PfbBR and PfbBF

PfbBR (SEQ ID NO 39): gccggttgcaacttgggtaagccccg (homologous to the sequence 1805657 to 1805632)

PfbBF (SEQ ID NO 40): tggcaggatcatccatgacagtaaaaacgg (homologous to the
30 sequence 1803996 to 1804025)

The chloramphenicol resistance cassette can then be eliminated. The plasmid pCP20 carrying recombinase FLP acting at the FRT sites of the chloramphenicol resistance

cassette is then introduced into the recombinant strains by electroporation. After a series of cultures at 42°C, the loss of the chloramphenicol resistance cassette is verified by a PCR analysis with the same oligonucleotides as those used previously. The strain retained is designated MG1655 $\Delta(pfkA, pfkB, udhA)$.

5 **a1) Introduction of the gene *yueD* coding for benzil reductase of *Bacillus cereus* into the strain $\Delta(pgi, udhA)$ or similarly $\Delta(pfkA, pfkB, udhA)$**

The gene *yueD* is cloned into the vector pTrc99A (Amersham-Pharmacia). The oligos YueDR and YueDF are used for the amplification of the gene from chromosomal DNA of *Bacillus cereus*.

10 YueDR (SEQ ID NO 41): CGTGAATTCtattcatcaattctaataa

with:

a region (lower case) homologous to the sequence (731 to 750) of the gene *yueD*,

a region (upper case) allowing the cleavage by the enzyme *EcoRI*.

15

YueDF (SEQ ID NO 42): ACGTTCatgAgAtacgttatcataacaggaac

with:

a region (lower case) homologous to the sequence (1 to 26) of the gene *yueD*,

a region (changed and added bases, upper case) allowing the cleavage by the enzyme *BspHI*.

20

The PCR product obtained is digested with the restriction enzymes *BspHI* and *EcoRI* and cloned into the vector pTrc99A that has been digested with *NcoI* and *EcoRI*. The resulting plasmid pYU1 is then introduced into the strain MG1655 $\Delta(pgi, udhA)$. The gene *yueD* codes for a NADPH-dependent benzil reductase that efficiently reduces 1-phenyl-1,2-propanedione ($k_{cat} = 165 \text{ min}^{-1}$; $K_m = 42 \text{ }\mu\text{M}$) but possesses a lower activity toward p-nitrobenzaldehyde ($k_{cat} = 1.2 \text{ min}^{-1}$; $K_m = 261 \text{ }\mu\text{M}$) (Maruyama, R. Nishizawa, M.; Itoi, Y.; Ito, S.; Inoue, M. (2002) The enzyme with benzil reductase activity is conserved from bacteria to mammals. *J. Biotechnology* **94**: 157-169).

30 **b) Culture and evolution of the modified strain on a minimal medium**

The maximal growth rate of the strain of *E. coli* [$\Delta(udhA, pgi) yueD$] obtained is evaluated on minimal medium ($\mu_{max} = 0.04$). In these conditions it is

much lower than that of the unmodified strain ($\mu_{\max} = 0.61$). It is then decided to add 1-phenyl-1,2-propanedione (co-substrate) to the minimal medium, and it is found that the modified strain is able to grow at a rate slightly inferior to that of the initial (*i.e.*, unmodified) strain on the same medium. It is then decided to seed (OD 5) a chemostat in a minimal medium containing p-nitrobenzaldehyde (co-substrate) with the modified strain; in these conditions the strain exhibits a growth rate similar to that of the modified strain grown on a minimal medium with no co-substrate. The chemostat is maintained for 1 to 5 weeks, while gradually increasing the dilution rate. The increase in the dilution rate can be performed stepwise or continuously. Glycerol stocks of the population contained in the chemostat are made up regularly.

d) Characterization of the evolved enzyme YueD

When the population can no longer adapt to the dilution rates imposed, it is considered that the selection is completed. Single colonies are isolated from the final evolved population (if necessary using one of the last glycerol stocks made up) and the kinetic characteristics of the evolved benzil reductase are evaluated, by comparison with the initial benzil reductase, using substrates 1-phenyl-1,2-propanedione and p-nitrobenzaldehyde. The k_{cat} value for the evolved benzil reductase is markedly improved for p-nitrobenzaldehyde, while its k_{cat} for 1-phenyl-1,2-propanedione is strongly depressed. Sequencing of the evolved clones demonstrates an accumulation of point mutations, which explains the altered substrate specificity of these enzymes.

REFERENCES

- Anderson, E.H. (1946), Growth requirements of virus-resistant mutants of *Escherichia coli* strain "B" *Proc. Natl. Acad. Sci. USA* **32**:120-128.
- A Baudin, O Ozier-Kalogeropoulos, A Denouel, F Lacroute, and C Cullin (1993), A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*, *Nucl. Acids Res.*, **21**: 3329-3330, 1993.
- Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD. (1998), Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*. **14**:115-32.

Datsenko, K.A.; Wanner, B.L. (2000), One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**: 6640-6645.

5 Miller, 1992. A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sambrook *et al.* (1989), Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.

10 Schaefer, U., Boos, W., Takors, R., Weuster-Botz, D. (1999), Automated sampling device for monitoring intracellular metabolite dynamics, *Anal. Biochem.* **270**: 88-96.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793-1808.